DESCRIPTION

METHOD FOR DIAGNOSING OR PREDICTING SUSCEPTIBILITY TO OPTIC NEUROPATHY

TECHNICAL FIELD

The present invention relates to a set of genetic polymorphisms linked to optic neuropathy.

BACKGROUND ART

Glaucoma is a major cause of blindness worldwide, and estimated approximately 67 million people suffered from some form of glaucoma. The majority of cases occur as late adult onset (typically over age 40 years) of primary open-angle glaucoma (POAG), which is the most common form of glaucoma and affects approximately 2% in white population and 7% of black population over 40 years old. POAG results in a characteristic visual field changes corresponding to the excavation of the optic disc that is usually associated with an elevation of intraocular pressure (IOP). Normal-tension glaucoma (NTG) is a form of open-angle glaucoma in which typical glaucomatous cupping of the optic nerve head and visual field loss are present but in which there is no evidence of increased IOP over 21 mm Hg at all times. In Japan, prevalence of glaucoma is approximately 3.5 % over 40 years old: POAG 0.58 % and NTG 2.04 %. Prevalence of NTG

in Japanese population is high compared with that in other populations. Glaucoma is a multifactorial disorder characterized by a progressive optic neuropathy associated with a specific visual field loss, and results from the interaction of multiple genes and environmental influences, although intraocular pressure (IOP) is a major risk factor for glaucoma.

Risk factors to develop glaucoma include high IOP, age, race, positive family history, myopia, the presence of diabetes or hypertension, and genetic factors. Although the exact pathogenesis of glaucomatous optic neuropathy is remains unclear, it is generally accepted that an increased IOP is a major risk factor. Current treatment for glaucoma consists of interventions which lower IOP. However, in some patients with glaucoma, NTG or advanced stage of POAG, reduction of IOP does not prevent the progression of the disease, indicating that factors other than an increased IOP may be involved in the development or progress of glaucoma.

POAG and NTG are a heterogeneous group of conditions probably with different multi-factorial etiologies resulting in the observed patterns of neuronal loss in the optic disk. The association between glaucoma and the presence of many systemic vascular diseases including low systemic blood pressure, nocturnal dips in blood pressure,

hypertension, migraine, vasospasm, and diabetes has been reported. The presence of optic disc hemorrhages in NTG patients suggests that vascular insufficiencies are deeply involved in the development and progression of NTG. A high percentage of patients with POAG receive a wide variety of medications for coexisting disorder. Especially, systemic hypertension was the most common disorder, occurring in 48% of the total population.

Glaucoma-like morphological changes have been reported in patients with Leber's hereditary optic neuropathy (LHON) at the atrophic stage and dominant optic atrophy (DAO). Recently, the inventor has reported optic disc excavation by a quantitative analysis using Heidelberg retinal tomography (HRT) in the atrophic stage of Japanese 15 patients with LHON harboring the 11778 mutation (Mashima Y et. al., Arch Clin Exp Ophthalmol 2003; 241:75-80, the contents of the cited reference are herein incorporated by reference). LHON is a maternally-transmitted eye disease that mainly affects young adult men. Approximately 70% of patients were male. This disease usually causes severe and permanent loss of vision resulting in a visual acuity of less than 0.1. Visual field defects are present as central or cecocentral scotomas. So far more than 20 point mutations of mitochondrial DNA (mtDNA) have been reported in LHON patients worldwide (Brown MD et. al., Clin Neurosci

1994; 2:138-145, the contents of the cited reference are herein incorporated by reference), and more than 80% of LHON patients carry one of three mtDNA mutations at nucleotide position 3460, 11778, or 14484 (Mackey DA et. al., Am J Hum Genet 1996; 59:481-485, the contents of the cited references are herein incorporated by reference). Although NTG patients were tested for the three LHON mutations of mtDNA nucleotide positions 3460, 11778 and 14484, no mutations and no defects in respiratory chain activity in skeletal muscle samples were detected (Brierley EJ et. al., Arch Ophthalmol 114:142-146 and Opial D et. al., Graefes Arch Clin Exp Ophthalmol 239:437-440, the contents of the cited references are herein incorporated by reference).

The major difference among LHON patients with one of these mtDNA mutations is in the clinical course. The 3460 and 14484 mutations are associated with better visual prognosis than the 11778 mutation which shows visual recovery rates of only 4% to 7% (OostraRJ et. al., J med Genet 1994;31:280-286, Riordan-Eva P et. al., Brain 1995; 118:319-337, Mashima Y et. al., Curr Eye Res 1998;17:403-408, the contents of the cited reference are herein incorporated by reference). However, visual recovery has been documented in some patients with the 11778 mutation and an age of onset in the low teens (Stone EM et. al., J

clin Meuro-Ophthalmol 1992; 12:10-14, Zhu D et. al., Am J Med Genet 1992; 42:173-179, Salmaggi A et. al., Intern J Neuroscience 1994; 77:261-266, Oostra RJ et. al., Clin Genet 1997; 51:388-393, Mashima Y et. al., Jpn J Ophthalmol 2002; 46:660-667, the contents of the cited references are herein incorporated by reference). Recovery of vision appears to be more likely when visual deterioration begins at an early age, even in patients with the 11778 mutation.

The clinical variability of LHON patients, which includes age at onset, male predilection, incomplete penetrance, and visual recovery, suggests that the disease most likely results from polygenic or multifactorial mechanisms, possibly involving environmental stressors, X-chromosomal loci, and other mtDNA mutations (Man PYW et. al., J Med Genet 2002; 39:162-169, the contents of the cited reference are herein incorporated by reference). However, attempts to identify a relevant locus on the X-chromosome have not been successful (Chalmers RM et. al., Am J Hum Genet 1996;59:103-108 and Pegoraro E et. al., Am J Med Genet 2003;119A:37-40, the contents of the cited reference are herein incorporated by reference). So-called "secondary LHON mutations" are more frequently found in European LHON patients than in unaffected Europeans and are polymorphisms linked to the European haplotype J. These polymorphisms are not strong autonomous risk factors (Brown

MD et. al., Am J Hum Genet 1997;60:381-387 and Torroni A et. al., Am J Hum Genet 1997;60:1107-1121, the contents of the cited reference are herein incorporated by reference).

Thus, the primary mutations are the major risk factors in LHON, but additional etiologic factors that augment or modulate the pathogenic phenotypes appear to be necessary. Considerable evidence indicates that heavy alcohol and/or tobacco use increases the risk of optic neuropathy in LHON families (Smith PR et. al., Q J Med 1993;86:657-660, Chalmers RM et. al., Brain 1996;119:1481-1486 and Tsao K et. al., Br J Ophthalmol 1999;83:577-581, the contents of the cited reference are herein incorporated by reference), although one study did not find this association. Possible secondary genetic interactions are complex and not firmly established (Kerrison JB et. al., Am J Ophthalmol 2000;130:803-812, the contents of the cited reference are herein incorporated by reference).

Oxidative stress has been implicated in many disorders associated with mutations of mtDNA. A recent investigation in an animal model identified reactive oxygen species (ROS) as a likely factor in the pathogenesis of LHON (Qi X et. al., Invest Ophthalmol Vis Sci 2003;44:1088-1096, the contents of the cited reference are herein incorporated by reference). Additionally, the mtDNA LHON pathogenic mutations were found to predispose cells to Fas-

dependent apoptotic death *in vitro* (Danielson SR et. al.,J Biol Chem 2002;277:5810-5815, the contents of the cited reference are herein incorporated by reference). These findings implied that there must be some nuclear modifier genes involved for developing LHON.

SUMMARY OF THE INVENTION

The inventor has revealed that some known and unknown SNPs are linked to onset of optic neuropathy including glaucoma and Leber's disease and completed the instant invention.

Accordingly, the present invention provides a set of genetic polymorphisms being associated with optic neuropathy, which comprises at least one polymorphism selected from the group consisting of:

(1) AAG to AAT substitution at codon 198 of the Endothelin-1 gene (Lys198Asn);

(2) -1370T>G polymorphism of the Endothelin-1 gene promoter region;

(3) A138 insertion/deletion(A138I/D) polymorphism in exon 1 of the Endothelin-1 gene;

(4) +70C>G polymorphism in 3' non-coding region of the Endothelin receptor A gene;

(5) +1222C>T polymorphism of the Endothelin Receptor A gene;

(6) CAC to CAT substitution at codon 323 in exon 6 of the

Endothelin Receptor A gene (His323His);

(7) -231A>G polymorphism of the Endothelin Receptor A gene promoter region;

(8) CTG to CTA substitution at codon 277 in exon 4 of the Endothelin receptor B gene;

(9) 9099C>A polymorphism of the Mitochondrial gene;

(10) 9101T>G polymorphism of the Mitochondrial gene;

(11) 9101T>C polymorphism of the Mitochondrial gene;

(12) 9804G>A polymorphism of the Mitochondrial gene;

(13) 11778G>A polymorphism of the Mitochondrial gene;

(14) -713T>G polymorphism of the Angiotensin II type 1 receptor gene promoter region;

(16) 3123C>A polymorphism of the Angiotensin II type 2 receptor gene;

(25) CAA to CGA substitution at codon 192 of the Paraoxonase 1 gene (Gln192Arg);

(26) TTG to ATG substitution at codon 55 of the Paraoxonase 1 gene (Leu55Met);

(27) CGG to CAG substitution at codon 144 of the Noelin 2 gene (Arg144Gln);

(32) GGA to CGA substitution at codon 389 of the β1 adrenergic receptor gene (Gly389Arg);

(35) 1105T>C polymorphism of the Myocilin gene (Phe369Leu);

(36) 412G>A polymorphism of the Optineurin gene;

(37) 1402C>T polymorphism of the E-Selectin gene;

(38) The combination of polymorphisms of -857C>T of the Tumor necrosis factor α gene promoter region and 412G>A of the Optineurin gene;

(39) The combination of polymorphisms of -863C>A of the Tumor necrosis factor α gene promoter region and 603T>A of the Optineurin gene

(40) CGC to CCC substitution at codon 72 of the TP53 gene (Arg72Pro);

(41) TAC to CAC substitution at codon 113 of the Microsomal epoxide hydrase1 gene (Tyr113His);

(42) -110A>C polymorphism of the Heatshock protein 70-1 gene promoter region;

(43) -338C>A polymorphism of the Endothelin converting enzyme gene promoter region;

(44) -670A>G polymorphism of the CD95 gene promoter region;

(45) AAG to AAA substitution at codon 119 of the Microsomal epoxide hydrase 1 gene(Lys119Lys);

(47) GGA to AGA substitution at codon 16 of the β2 adrenergic receptor gene (Gly16Arg); and

(48) CAA to GAA substitution at codon 27 of the β2 adrenergic receptor gene (Gln27Glu).

In addition, the present invention also provides a method for diagnosing or predicting susceptibility to optic neuropathy in a human subject, which comprising the steps of:

i) obtaining a biological sample from the subject,

ii) determining genotype of the sample in respect of the set of the polymorphisms defined as above, and

iii) diagnosing or predicting susceptibility to optic neuropathy in the subject based on the genotype.

According to the present invention, the optic neuropathy may preferably be glaucoma or Laber's disease. The polymorphism (1)-(39) and (42)-(48) may be used especially for glaucoma. Among them, those (1), (2), (5)-(7), (16), (26), (32), (43) and (45) may be used especially for normal tension glaucoma and those (4), (14), (25), (35), (36), (38), (42), (44), (47)-(48) may be used especially for primary open angle glaucoma. The polymorphisms (40) and (41) may be used especially for Laber's disease.

According to the present invention, the set of polymorphisms may further comprise at least one other polymorphism which has been known to be associated with optic neuropathy.

In another aspect of the present invention, a kit for diagnosing or predicting susceptibility to optic neuropathy in a human subject which comprises primer set and/or probe suitable for determining genotype in respect of the set of genetic polymorphisms defined as above.

In further aspect of the present invention, neuly identified SNPs are provided in Mitocondorial gene,

Myocilin gene and Noelin 2 gene. Accordingly, the present invention encompass nucleotide fragment covering those SNPs. In general, in order to determin genotype in respect of said SNP, 90 or more contignous nucleotide sequence containing the SNP may be required. Namely, an isolated polynucleotide consisting of a segment of the sequence:

8881 tctaagatta aaaatgccct agcccacttc ttaccacaag gcacacctac acccctttatc

8941 cccatactag ttattatcga aaccatcagc ctactcattc aaccaatagc cctggccgta

9001 cgcctaaccg ctaacattac tgcaggccac ctactcatgc acctaattgg aagcgccacc

9061 ctagcaatat caaccattaa ccttccctct acacttatca tcttcacaat tctaattcta

9121 ctgactatcc tagaaatcgc tgtcgcctta atccaagcct acgttttcac acttctagta

9181 agcctctacc tgcacgacaa cacataatga cccaccaatc acatgcctat catatagtaa

wherein the segment comprises at least 90 contignuous nucleotide, and the at least 90 contignuous nucleotide includes position 9099 of the sequence, and wherein position 9099 of the sequence is A or an isolated polynucleotide which is entirely complementary to the above segment; or

wherein the segment comprises at least 90 contignuous nucleotide, and the at least 90 contignuous nucleotide includes position 9101 of the sequence, and wherein position 9101 of the sequence is G; or

an isolated polynucleotide which is entirely complementary to either of the above segment.

The present invention further provides an isolated

polynucleotide consisting of a segment of the sequence:

301 actggaaagc acgggtgctg tggtgtactc ggggagcctc tatttccagg gcgctgagtc

361 cagaactgtc ataagatatg agctgaatac cgagacagtg aaggctgaga aggaaatccc

421 tggagctggc taccacggac agttcccgta ttcttggggt ggctacacgg acattgactt

481 ggctgtggat gaagcaggcc tctgggtcat ttacagcacc gatgaggcca aaggtgccat

541 tgtcctctcc aaactgaacc cagagaatct ggaactcgaa caaacctggg agacaaacat

wherein the segment comprises at least 90 contignuous nucleotide, and the at least 90 contignuous nucleotide includes codon 369, which is corresponding to the underlined nucleotides of the sequence, and wherein codon 369 is substituted such that it codes for Leu, or an isolated polynucleotide which is entirely complementary to the above segment.

The present invention further provides an isolated polynucleotide consisting of a segment of the sequence:

79741 ttagttccta caatggagtc atgtctggga agaatctagg gtccaatatg agccacatgt

79801 caagggccag gtgtgcatca aagacaaagg gtgaagttat gagtcagagg ttggagtcat

79861 gtctgggtca aaggccaggg gtcaggcttg gccatggttc catcttgatg cacaggagct

79921 gaaggacagg atgacggaac tgttgcccct gagctcggtc ctggagcagt acaaggcaga

79981 cacgcggacc attgtacgct tgcgggagga ggtgaggaat ctctccggca gtctggcggc

80041 cattcaggag gagatgggtg cctacgggta tgaggacctg cagcaacggg tgatggccct

80101 ggaggcccgg ctccacgcct gcgcccagaa gctgggtatg ccttggccct tgaccctgac

80161 ccctgatctc tgactgccac acccaactcc agtatcacct gtttgtgcct agaagctgga

80221 cacagttttg acctctaact tttaaacctc aacccttgac cttcctacct aaggctacac

wherein the segment comprises at least 90 contignuous nucleotide, and the at least 90 contignuous nucleotide includes codon 144, which is corresponding to the underlined nucleotides of the sequence, and wherein codon 144 is substituted such that it codes for Gln, or an isolated polynucleotide which is entirely complementary to the above segment.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 represents correlation of clinical Characteristics of NTG Patients with AT2R 3123C>A Polymorphism and ACE I/D Polymorphism

Fig. 2 represents DHPLC tracing patterns in the Exon3C of the *MYOC* gene.

Fig. 3 represents novel missense mutation, Phe369Leu detected in exon 3 of the MYOC gene.

Fig. 4 represents a DHPLC tracing of MYOC gene from a patient with POAG.

Fig. 5A represents the IOP after oral candesartan cilexetil or placebo.

Fig. 5B represents the ocular perfusion pressure after oral candesartan cilexetil or placebo

Fig. 5C represents the IOP after oral candesartan cilexetil in each of the 15 subjects.

PREFERRED EMBODIMENT OF THE INVENTION

In the present specification and claims, "genetic

polymorphism" means genomic diversity between individuals at a locus. Genetic polymorphism may be single nucleotide substitution called as "Single nucleotide polymorphisms" or "SNPs" as well as those consisting of plural nucleotides. The genetic polymorphism may or may not be those affect on the phenotype of the individual. In addition, a nucleotide sequence of an individual is different from the corresponding wild type sequence, i.e., having insertion, deletion or substitution on the wild type sequence, said nucleotidesequence is called as "genetic mutant" and the genetic mutant is also included in "polymorphic variant" according to the present invention.

In the present specification and claims, expression like "9099C>A" or "C9099A" means that the gene has a polymorphsm at position 9099, that is, there are two alleles of the gene and the one has cytosine or C and the other has adenine or A at 9099 (bi-allelic). It does not necessarily mean the frequent allele has C whereas the rare allele has A at said position.

The expression like "Gln192Arg" represents an amino acid substitution due to the base substitution in the gene coding for the amino acid sequence. For example, Gln192Arg represents Glycine at codon 192, i.e. amino acid number 192, is replaced with Arginine or Arg. This also means that there are polymorphic variants of the protein wherein the

amino acid at codon 192 is Gln or Arg.

According to the present invention, determining genotype in respect of the genetic polymorphisms may be carried out by every single polymorphism, or plurality or all polymorphisms may be determined at the same time.

In the present invention, the method for diagnosing or predicting susceptibility to optic neuropathy in a human subject which comprises determining genotype in respect of the set of genetic polymorphism of which relationship with optic neuropathy is newly reported in this application. In addition to the genetic polymorphism identified as being linked to optic neuropathy by the instant invention, any other polymorphism which had been revealed as being linked to optic neuropathy may be detected together. By employing plural genetic polymorphisms linked to optic neuropathy, the diagnostic probability can be improved.

According to the present invention, the method used for determining genotype in respect of the genetic polymorphisms is not limited and may be any of those known to the art. Representative method for determining genotype in respect of the genetic polymorphisms include polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) analysis, polymerase chain reaction followed by single strand conformation polymorphism (PCR-SSCP) analysis, ASO hybridization analysis, direct sequencing analysys,

ARMS analysis, DGGE analysis, RNaseA cleaving analysis, chemical restriction analysis, DPL analysis, TaqMan® PCR analysis, Invader® assay, MALDI-TOF/MS analysis, TDI analysis, single nucleotide extension assay, WAVE assay, one molecular fluorescent detection assay. According to the present invention, the detection method may be one of those or combination of two or more.

According to the present invention, biological sample to be used for detecting the genetic polymorphism is not specifically limited and may be hair, blood, saliva, lymph fluid, respiratory tract mucosa, cultured cells and urine.

In the specification and claims, "diagnosing or predicting susceptibility to optic neuropathy" includes not only diagnosing onset of optic neuropathy but also determining risk factors which hasten onset of the disease as well as accelerate the disease progresses.

According to the present invention, kits for detecting the genetic polymorphism as well as protein polymorphism identified as above are also provided. Said kits may comprise primers and/or probes which are specifically designed for detecting the above-identified genetic polymorphisms; antibodies for detecting the above-identified protein polymorphism. According to the present invention, said kit may be used for diagnosing or predicting susceptibility to optic neuropathy.

In the present specification and claims, the term "primer" denotes a specific oligonucleotide sequence which is complementary to a part of the target nucleotide sequence and used to hybridize to the target nucleotide sequence. A primer serves as an initiation point for nucleotide polymerization catalyzed by either DNA polymerase, RNA polymerase or reverse transcriptase.

The term "probe" denotes a defined nucleic acid segment which can be used to identify a specific polynucleotide sequence present in samples or confirming target DNA or RNA in a gene modifying process, said nucleic acid segment comprising a nucleotide sequence complementary to the specific polynucleotide sequence to be identified.

According to the present invention, primers and probes may be designed based on the targeted sequence so that they are specific to the position at which the targeted polymorphism is expected and/or surrounding sequence of the position so long as they are not identical to some other genes, i.e. it is necessary not to be repeating sequence nor palindrome sequence.

According to the present invention, genetic polymorphisms which are linked to optic neuropathy, especially glaucoma and Leber's disease are identified. Based on the findings, the genotype in respect of the genetic polymorphisms of a biological sample obtained from

an individual is determined and based on thus obtained genotype, onset of the disease or predicted risk for onset of the disease can be determined.

In addition to the polymorphisms identified (1)-(48) as above, genotypes in respect of some other genetic polymorphisms which had been known to the art being highly associated with optic neuropathy may be determined for improved relaiability of the diagnosis or prediction.

For example, two types of genetic polymorphisms in myocilin as well as optineurin genes have been revealed by the inventor to be associated with onset of primary open-angle glaucoma. In addition to the two genes, 4 other genetic polymorphisms including mutations had been identified to be associated with primary open-angle glaucoma. Almost 100% of the subjects having both the risk genotype in respect of the genetic polymorphisms of the present invention and of those already known to the art may develop glaucoma. That is, the set of the genetic polymorphisms will be useful for precrinical test.

In regard of some SNPS, the inventor confirmed correlation with optic neuropathy in a specific group, such as race or sex. Accordingly, said SNPs may prefereably be used for diagnosing or predicting the risk for optic neuropathy in the specified group.

Further, statical analysis of the genotyp in respect

of the set of polymorphisms may provide useful information such as predictive age of onset, predictive association with lifestyle-related diseases, predictive association with symptom factors. In addition, effect of some medical treatments may also be predictable based on the information.

According to the present invention, predicting susceptibility to optic neuropathy can be carried out before onset of the disease based on the genotype, and the subject can receive advice on how to remove the risk factor, for example, to improve life style or alter the environment. In addition, it may possible to receive an early treatment such as reduction of the risk gene. an appropriate treatment can be started earlier. Consequently, those "order made treatment" can reduce the risk for vision loss.

For example, in case a subject has the genotype linked to high risk for onset of optic neuropathy, inhibition of onset, reduction of the risk of onset or relief of symptoms can be expected by introducing to the subject the genotype linked to low risk for onset and expressing the same. Further, anti sence to the mRNA of the allele of high risk for onset of optic neuropathy or RNAi method may be used for inhibiting expression of the high risk allele.

In another aspect, based on the genotype determination in respect of the set of polymolphisms shown in the present

invention, genetic etiology of optic neuropathy may be revealed and thus obtained etiology may be useful for development of novel medical agents.

Further, by combining genotype information which is associated with optic neuropathy obtained by the present invention and the other genotype information which is associated with life style diseases and the like, comprehensive risk for age-related, life-style related diseases can be predicted and used for high quality of life.

The present invention will be further illustrated by means of the examples shown below. It is to be expressly understood, however, that the examples are for purpose of illustration only and is not intended to limit of the scope of the invention.

EXAMPLE 1 Genetic Variants of TP53 and EPHX1 in Leber's Hereditary Optic Neuropathy and their Relationship to Age at Onset

Purpose: To determine whether genetic polymorphisms of the genes for oxidative stress and apoptosis cause the clinical variability in patients with Leber's hereditary optic neuropathy (LHON).

MATERIALS AND METHODS

Patients

We studied 86 unrelated Japanese patients with LHON

carrying the 11778 mutation with homoplasmy. Their mtDNA mutation was confirmed by polymerase chain reaction followed by a restriction-enzyme assay which revealed concordant gain of the *Mae*III site (Mashima Y et. al., Curr Eye Res 1998;17:403-408, the contents of the cited reference are herein incorporated by reference).

The mean age at the onset of visual loss in 86 LHON patients was 25.1 ± 13.0 years with a range 3 to 65 years.

Genomic DNA Extraction and Genotyping

DNA was extracted from peripheral blood leukocytes by the SDS-proteinase K and phenol/chloroform extraction method. Polymorphisms were examined in the oxidative stress-related gene, microsomal epoxide hydrolase (EPHX1) (Kimura K et. al., Am J Ophthalmol 2000;130: 769-773, the contents of the cited reference are herein incorporated by reference).,-and the apoptosis-related gene, Arg72Pro in-TP53 (Ara S et. al., Nucleic Acids Res 1990; 18:4961, the contents of the cited reference are herein incorporated by reference).

Each polymorphism was identified using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) techniques (**Table 1**).

Table 1. Primer sequences, product size, and annealing temperatures

Gene		Primer sequences	Product Size (bp)	Annealing Temperature (℃)	Restriction Enzyme
TP53	F	TTG CCG TCC CAA GCA ATG GAT GA	199	60.0	Acc II
	R	TCT GGG AAG GGA CAG AAG ATG AC			
EPHX1	F	GAT CGA TAA GTT CCG TTT CAC C	165	56.0	EcoR V
	R	TCA ATC TTA GTC TTG AAG TGA GGA T			

RESULTS

The associations between age at onset and the polymorphisms were presented in **Table 2-1 and Table 2-2.**

Table 2-1. Association between age at onset and TP53 (Arg72Pro) and EPHX1 (Tyr113His) gene polymorphism in Leber's hereditary optic neuropathy

Gene	Genotype		P
TP53 (Arg72Pro) Age at onset	Arg/Arg 20.7±10.6(n=35)	Arg/Pro + Pro/Pro 28.1±13.8(n=51)	0.009
EPHX1 (Tyr113His) Age at onset	Tyr/Tyr + Tyr/His 27.9±13.9(n=45)	His/His 22.1±11.4(n=41)	0.038

P Value for t-test

Table 2-2. Association between age at onset and TP53 (Arg/Arg) and EPHX1 (His/His) gene polymorphism in Leber's hereditary optic neuropathy

Group 1	Group 2	Group 3	*P*
Arg/Arg and His/His	Arg/Arg or His/His	others	
17.7±9.3 (n=19)	25.3±11.3 (n=38)	29.8±15.1 (n=29)	0.0044

P value for Kruskal-Wallis

Group 1: Patients who have Arg/Arg at codon 72 in TP53 and His/His at codon 113 in EPHX1

Group 2: Patients who have Arg/Arg at codon 72 in TP53 but not His/His at codon 113 in EPHX1, or His/His at codon 113 in EPHX1 but not Arg/Arg at codon 72 in TP53

Group 3: Patients other than Groups 1 and 2

As shown in Table 2-1, the codon 72 genotype in TP53 and the codon 113 genotype in EPHX1 were significantly

associated with younger age at onset of Leber's hereditary optic neuropathy.

As shown in Table 2-2, the co-existence of the Codon 72 genotype in TP53 and the codon 113 genotype in EPHX1 were significantly associated with younger age at onset of Leber's hereditary optic neuropathy.

These results indicated that detection of the Arg/Arg homozygote in TP53 and His/His homozygote in EPHX1 make possible the early diagnosis and early treatment of Leber's hereditary optic neuropathy.

These results also indicated that the Codon 72 polymorphism may interact with mitochondrial dysfunction to influence disease expression. Individual variations may exist in the apoptotic response that is correlated with the polymorphism at codon 72 of p53. Bonafe et al (Biochem Biophys Res Commun 2002;299:539-541.). reported that cultured cells from healthy subjects carrying the Arg/Arg genotype underwent more extensive apoptosis than cells from Arg/Pro subjects in response to the cytotoxic drug cytosine arabinoside. Thus, naturally occurring genetic variability at the p53 gene could partly explain individual differences in *in vivo* susceptibility of cells to a chemotherapeutic drug. Dumount et al (Nat Genet 2003;33:357-365). reported that the Arg72 variant was more efficient than the Pro72 variant at inducing apoptosis, with at least one mechanism

underlying this greater efficiency being enhanced localization of Arg72 variant to mitochondria in tumor cells. The synthetic p53 inhibitors might be highly effective in treating LHON in which neurons died by apoptosis triggered by mitochondrial impairment and oxidative stress.

Partial nucleotide sequences for EPHX1 and TP53 genes containing the targeted polymorphism are as follows:

EPHX1 Tyr113His Codon 113 (underlined) (TAC to CAC change)

181 tgctgggctt tgccatctac tggttcatct cccgggacaa agaggaaact ttgccacttg
241 aagatgggtg gtgggggcca ggcacgaggt ccgcagccag ggaggacgac agcatccgcc
301 ctttcaaggt ggaaacgtca gatgaggaga tccacgactt acaccagagg atcgataagt
361 tccgtttcac cccacctttg gaggacagct gcttccacta tggcttcaac tccaactacc
421 tgaagaaagt catctcctac tggcggaatg aatttgactg gaagaagcag gtggagattc
481 tcaacagata ccctcacttc aagactaaaa ttgaagggct ggacatccac ttcatccacg
541 tgaagccccc ccagctgccc gcaggccata ccccgaagcc cttgctgatg gtgaacggct
601 ggcccggctc tttctacgag ttttataaga tcatcccact cctgactgac cccaagaacc
661 atggcctgag cgatgagcac gtttttgaag tcatctgccc ttccatccct ggctatggct
721 tctcagaggc atcctccaag aaggggttca actcggtggc caccgccagg atcttttaca

TP 53 Codon 72(underlined): CGC(Arg) to CCC(Pro),

13081 gcaggcccac caccccgacc ccaaccccag ccccctagca gagacctgtg ggaagcgaaa
13141 attccatggg actgactttc tgctcttgtc tttcagactt cctgaaaaca acgttctggt
13201 aaggacaagg gttgggctgg ggacctggag ggctggggac ctggagggct ggggggctgg
13261 ggggctgagg acctggtcct ctgactgctc ttttcaccca tctacagtcc ccc*ttgccgt*

13321 *cccaagcaat ggatga*atttg atgctgtccc cggacgatat tgaacaatgg ttcactgaag

13381 acccaggtcc agatgaagct cccagaatgc cagaggctgc tccccgccgtg gcccctgcac

13441 cagcagctcc tacaccggcg gcccctgcac cagccccctc ctggcccctg *tcatcttctg*

13501 *tcccttccca gaaaacctac* cagggcagct acggtttccg tctgggcttc ttgcattctg

13561 ggacagccaa gtctgtgact tgcacggtca gttgccctga ggggctggct tccatgagac

13621 ttcaatgcct ggccgtatcc ccctgcattt cttttgtttg gaactttggg attcctcttc

13681 acccctttggc ttcctgtcag tgtttttttа tagtttaccc acttaatgtg tgatctctga

13741 ctcctgtccc aaagttgaat attccccccct tgaatttggg cttttatcca tcccatcaca

13801 ccctcagcat ctctcctggg gatgcagaac ttttcttttt cttcatccac gtgtattcct

Example 2 Mitochondrial DNA mutations related with Leber's hereditary optic neuropathy in primary open-angle glaucoma and normal-tension glaucoma

Materials and Methods

Patients

A total of 651 blood samples were collected at seven institutions in Japan. There were 201 POAG patients, 232 NTG patients, and 218 normal controls, and none of the subjects was related to others in this study.

The mean age at the time of examination was 61.2 ± 16.0 years in POAG, 58.8 ± 13.6 years in NTG, and 70.6 ± 10.9 years in the control subjects. The mean age of the control subjects was significantly older than that of POAG patients (P <0.001) and the NTG patients (P <0.001). We

purposely selected older control subjects to reduce the probability that a subset of them would eventually develop glaucoma. There were 112 (55.7%) men in the POAG group, 108 (46.6%) in the NTG group, and 89 (40.8%) in the control group.

Patients were considered to have POAG if they had a normal open-angle, a cup-disc ratio greater than 0.7 with typical glaucomatous visual field loss on either Goldmann or Humphrey perimetry, and the absence of ocular, rhinologic, neurological, or systemic disorders which might be responsible for the optic nerve damage. Patients with NTG had an IOP of 21 mmHg or lower. Patients with exfoliative glaucoma, pigmentary glaucoma, and corticosteroid-induced glaucoma were excluded.

Two-hundred-eighteen control samples were obtained from Japanese subjects who had no known eye abnormalities except for cataracts. These subjects were older than 40 years, had IOPs below 21 mm Hg, had normal optic discs, and no family history of glaucoma.

Detection of mtDNA Mutations by Invader® Assay

Genomic DNA was isolated from peripheral blood lymphocytes by standard methods of phenol-chloroform extraction.

The primary probes (wild and mutant probes) and Invader® oligonucleotides (Invader® probe) used to detect

the six mtDNA mutations (G3460A, T9101C, G9804A, G11778A, T14484C, and T14498C) by the Invader® assay are shown in Table 3.

Table 3. The oligonucleotide sequence of wild type, mutant, and Invader probes with Invader assay to detect mutation of mtD

Nucleotide	Target	Probe	Sequence	Tm	Dye
G3460A	Anti-sense	Wild	Flap sequence-gccataaaactcttcacca	63.2	RED
		Mutant	Flap sequence-accataaaactcttcaccaaa	63.3	FAM
		Invader	cctacgggctactacaacccttcgctgact	77.7	
T9101C	sense	Wild	Flap sequence-atgataagtgtagagggaagg	64.1	FAM
		Mutant	Flap sequence-gtgataagtgtagagggaag	62.2	RED
		Invader	ggcgacagcgatttctaggatagtcagtagaattagaattgtgaagT	76.8	
G9804A	anti-sense	Wild	Flap sequence-gcccacaggcttcca	63.7	FAM
		Mutant	Flap sequence-accacaggcttccac	63.7	RED
		Invader	catttccgacggcatctacggctcaacatttttgtaT	76.7	
G11778A	Anti-sense	Wild	Flap sequence-gcatcataatcctctctcaag	63.5	RED
		Mutant	Flap sequence-acatcataatccctctctcaag	62.2	FAM
		Invader	gcctagcaaactcaaactacgaacgcactcacagtct	77.7	
T14484C	Sense	Wild	Flap sequence-atggttgtctttggatatactac	63.4	FAM
		Mutant	Flap sequence-gtggttgtctttggatatacta	62.8	RED
		Invader	ttttgggggaggttatatggtttaatagttttttaatttattatttaggggaatgt	76.0	
T14498C	sense	Wild	Flap sequence-atttaggggggaatgatggt	64.0	FAM
		Mutant	Flap sequence-gtttaggggggaatgatgg	62.7	RED
		Invader	tgttattattctgaatttggggggaggttatatggtttaatagttttttttaatttT	74.1	

Invader® assay FRET-detection 256-well plates (Third Wave Technologies, Inc, Madison, WI) contains the generic components of an Invader® assay (Cleavase® enzyme VIII, FRET probes, MOPS buffer, and polyethylene glycol) dried in each of the individual wells. The biplex format of the Invader® assay enabled simultaneous detection of two DNA sequences in a single well.

The detail method was described previously. In brief, 8 µl of the primary probe/Invader®/mixture and total DNA (10 ng) samples were added to each well of a 96-well plate, and were denatured by incubation at 95° C for 10 min. After 15 µl of mineral oil (Sigma, St. Louis, MO) was overlaid on all reaction wells, the plate was incubated isothermally at 63° C for 2 hours in a PTC-100 thermal cycler (MJ Research, Waltham, MA) and then kept at 4° C until fluorescence measurements. The fluorescence intensities were measured on a CytoFlour 4000 fluorescence plate reader (Applied Biosystems, Foster City, CA) with excitation at 485 nm/20 nm (wavelength/ bandwidth) and emission at 530 nm/25 nm for FAM dye; excitation at 560 nm/20 nm and emission at 620 nm/40 nm for Redmond RED (RED) dye. Each samples was tested in duplicate in the same plate and two fluorescence measurements were performed in each plate. Thus, four measurements were obtained for each sample and they were averaged.

Direct DNA Sequencing

To detect mutations by direct sequencing, the PCR products were first purified with the QIAquick PCR Purification Kit (QIAGEN, Valenica, CA, USA) to remove unreacted primers and precursors. The sequencing reactions were then performed using the ABI PRISM BigDye Terminator (v.3.1) Cycle Sequencing Kit, according to the manufacturer's protocol (Applied Biosystems). The data were collected by the ABI PRISM 310 Genetic Analyzer and analyzed by the ABI PRISM sequencing analysis program (v.3.7).

Table 4. Primer sequences

mutation		Primer Sequences (5' to 3')
3460	F	CAG TCA GAG GTT CAA TTC CTC
	R	TGG GGA GGG GGG TTC ATA GTA
11778	F	GGC GCA GTC ATT CTC ATA AT
	R	AAG TAG GAG AGT GAT ATT TG
14484	F	none
	R	GCT TTG TTT CTG TTG AGT GT
9101	F	AAA ATG CCC TAG CCC ACT TC
	R	GTC ATT ATG TGT TGT CGT GC
9804	F	CAC ATC CGT ATT ACT CGC AT
	R	CGG ATG AAG CAG ATA GTG AG

RESULTS

A total of 651 Japanese subjects were studied. When a nucleotide substitution is located within a primary probe or an invader probe, the examined cases showed no reaction to both probes by Invader assay. In such cases, direct

sequence analysis showed single nucleotide polymorphisms (SNPs) at the nucleotide position of 9099, 9101, 9102, 9797, and 9815.

As shown in **Table 5**, 7 patients including 5 females and 2 males harbored 5 mutations of mtDNA, and have not developed LHON. Two patients (Cases 1 and 2) harbored novel amino acid changes which have not been to associated with LHON, and 5 patients (Cases 3 to 7) harbored LHON mutations.

These mtDNA mutations were not detected in normal controls.

Table 5.

Case	mtDNA mutation	Patient
1	C9099A mutation (Ile to Met)	POAG (Male)
2	T9101G mutation (Ile to Ser)	POAG (Female)
3	T9101C mutation (Ile to Thr)	POAG (Female)
4	G9804A mutation (Ala to Thr)	POAG (Male)
5	G9804A mutation (Ala to Thr)	NTG (Female)
6	G11778A mutation (Arg to His) heteroplasmy 80%	POAG (Female)
7	G11778A mutation (Arg to His) heteroplasmy 15%	NTG (Male)

As described above, we found 5 mtDNA mutations including 2 novel mtDNA mutations in glaucoma patients. These results indicated that mtDNA mutations is one of the risk factor to develop or progress the glaucoma, and detection of the mtDNA mutations makes possible the early diagnosis and early treatment of glaucoma.

Partial nucleotide sequences of mitochondrial gene containing the targeted mutations/polymorphism are as

follows:

C9099A, T9101G (underlined)

8881 tctaagatta aaaatgccct agcccacttc ttaccacaag gcacacctac acccttatc

8941 cccatactag ttattatcga aaccatcagc ctactcattc aaccaatagc cctggccgta

9001 cgcctaaccg ctaacattac tgcaggccac ctactcatgc acctaattgg aagcgccacc

9061 ctagcaatat caaccattaa ccttccctct acacttatca tcttcacaat tctaattcta

9121 ctgactatcc tagaaatcgc tgtcgcctta atccaagcct acgttttcac acttctagta

9181 agcctctacc tgcacgacaa cacataatga cccaccaatc acatgcctat catatagtaa

G9804A (underlined)

9541 taggagggca ctggccccca acaggcatca ccccgctaaa tcccctagaa gtcccactcc

9601 taaacacatc cgtattactc gcatcaggag tatcaatcac ctgagctcac catagtctaa

9661 tagaaaacaa ccgaaaccaa ataattcaag cactgcttat tacaatttta ctgggtctct

9721 attttaccct cctacaagcc tcagagtact tcgagtctcc cttcaccatt tccgacggca

9781 tctacggctc aacatttttt gtagccacag gcttccacgg acttcacgtc attattggct

9841 caactttcct cactatctgc ttcatccgcc aactaatatt tcactttaca tccaaacatc

9901 actttggctt cgaagccgcc gcctgatact ggcattttgt agatgtggtt tgactatttc

G11778A (underlined)

11641 agccctcgta gtaacagcca ttctcatcca aacccccctga agcttcaccg gcgcagtcat

11701 tctcataatc gcccacgggc ttacatcctc attactattc tgcctagcaa actcaaacta

11761 cgaacgcact cacagtcgca tcataatcct ctctcaagga cttcaaactc tactcccact

11821 aatagctttt tgatgacttc tagcaagcct cgctaacctc gccttacccc ccactattaa

11881 cctactggga gaactctctg tgctagtaac cacgttctcc tgatcaaata tcactctcct

11941 acttacagga ctcaacatac tagtcacagc cctatactcc ctctacatat ttaccacaac

12001 acaatggggc tcactcaccc accacattaa caacataaaa ccctcattca cacgagaaaa

Example 3 Gene polymorphisms of the renin-angiotensin aldosterone system associate with risk for developing primary open-angle glaucoma and normal-tension glaucoma

Purpose: Multiple environmental and genetic factors may be involved in pathogenesis of glaucoma. To predict genetic risk of glaucoma, an association study in gene polymorphisms of the renin-angiotensin-aldosterone (R-A-A) system was performed.

MATERIALS and METHODS

Patients and Control study subjects

A total of 551 blood samples were collected at seven institutes in Japan. They were 162 POAG patients, 193 NTG patients, and 196 normal subjects, and none of the subjects was related to others in this study.

The average age at examination was 58.8 ± 13.7 years in NTG, 62.0 ± 15.4 years in POAG, and 71.2 ± 10.4 years in normal subjects. The average age of the normal control subjects is significantly higher than NTG patients ($p < 0.001$) or POAG patients ($p < 0.001$), respectively. This could reduce the possibility that a subset will eventually develop glaucoma. The familial history was recorded in 66

(34.2%) out of 127 NTG patients and 49 (30.2%) out of 113 POAG patients. Male patients were 89 (46.1%) in NTG and 87 (53.7%) in POAG, and 77 (39.3%) in normal subjects.

One hundred ninety-six Japanese control samples were obtained from individuals who had no known eye abnormalities except cataract. These subjects were older than 40 years with IOP below 21 mmHg, no glaucomatous disc change, and no family history of glaucoma.

Genotyping

Seven genes and 10 polymorphisms in the R-A-A system were determined for each subject with glaucoma or normal Japanese control with renin (REN) I8-83G>A (Frossard PM et. al., Hypertens Res 1998;21:221-225, the contents of the cited reference are herein incorporated by reference), angiotensin II type 1 receptor (AT1R) 1166A>C, -521C>T, -713T>G (Nalogowska-Glosnicka K et. al., Med Sci Monit 2000;6:523-529 and Erdmann J et. al., Ann Hum Genet 1999;63:369-374, the contents of the cited reference are herein incorporated by reference), angiotensin II type 2 receptor (AT2R) 3123C>A (Katsuya T et. al., Mol Cell Endocrinol 1997;127:221-228, the contents of the cited reference are herein incorporated by reference), cytochrome P45011B1 (CYP11B1) -344T>C (Tsujita Y et. al., Hypertens Res 2001;24:105-109, the contents of the cited reference are herein incorporated by reference), and chymase (CYM)

3123C>A, were identified using by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). The angiotensin-converting enzyme (ACE) insertion/deletion (I/D) was determined only by PCR and agarose gel electrophoresis. To avoid the false determination of ACE/ID polymorphism, I allele specific amplification was carried out following the protocol of Lindpaintner et al (N Engl J Med 1995; 332: 706-711, the contents of the cited reference are herein incorporated by reference). Genomic DNA was isolated from peripheral blood lymphocytes by phenol-chloroform extraction. The primer sets and restriction enzymes used were listed in **Table 6.**

Table 6. Primer pair sequences used for PCR amplification and restriction enzymes of polymorphic sites in renin angiotensin system

Gene	Polymorphism	Primer sequences	Annealing temp	Product size	Restiction enzyme	Digested products
REN	I8-83G>A	TGAGGTTCGAGTCGGCCCCCT	68℃	250bp	*Mbo*I	G: 250bp
		TCGCCAAACAATGGCCACACAT				A: 171+79bp
ACE	I/D 1st step	GCCCTGCAGGTGTCTGCAGCATGT	63℃	D: 319bp		
		GGATGGCTCTCCCCGCCTTGTCTC		I: 597bp		
	2nd step	TGCCAGCACAGCGCCCCCACTAC	67℃	D/D: no product		
		TCGCCAGCCCTCCCATGCCCATAA		I: 335bp		
AT1	1166A>C	GAGGTTGAGTGACATGTTCGAAAC	60℃	253bp	*Dde*I	A: 253bp
		CGTCAACTCTGTCTAATGCAAAATGT				C: 155+98bp
	-521C>T	CGTGATGTCTTTATCTGGTTTTG	60℃	270bp	*Ssp*I	C: 270bp
		CGAACTTTGGTAATACAGTTGTGG				T: 144+126bp
	-713T>G	AAACTACAGTCACCCTACTCACCT	55℃	292bp	*Hin*fI	T: 170+122bp
		TTCTTCACAAACTCTTCCAA				G: 292bp
AT2	3123C>A	GGATTCAGATTTCTCTTTGAA	53℃	340bp	*Alu*I	C: 340bp
		GCATAGGAGTATGATTTAATC				A: 227+113bp
CYP11B1	-344C>T	CAGGAGGATGAGCAGGCAGAGCACAG	63℃	404 bp	*Bae*III	C: 333bp + 71bp
		CTCACCCAGGAACCTGCTCTGGAAACATA				T: 404 bp
CMA	-1903A>G	GGAAATGTGAGCAGATAGTGCAGTC	51℃	285bp	*Bst*XI	A: 285bp
		AATCCGGAGCTGGAGAACTCTTGTC				G: 195+90bp

The genotyping angiotensinogen (AGT) T174M, M235T was determined using by Invader assay® (Lyamichev V et. al., Nat Biotechnol 1999;17:292-296, the contents of the cited reference are herein incorporated by reference).

RESULTS

Genotype distribution of R-A-A system in Japanese population

Of 10 polymorphisms in R-A-A system, two showed a significantly difference in frequencies of genotypes: AT1R/-713T>G for POAG, and AT2/3123C>A for NTG (Table 7). A 3123C>A polymorphism was associated with only female patients with NTG.

A frequency of homozygous G genotype (GG) in AT1R/-713T>G polymorphism was significantly higher (p=0.04 for TT+TG v GG) in POAG patients (4.2%) than in controls (0.5%). A frequency of CA+AA genotypes in AT2R/3123C>A polymorphism was significantly higher (p=0.011 for CC v CA+AA) in female patients with NTG (70.8%) than in female controls (55.0%).

Table 7. Association between glaucoma (POAG and NTG) and gene polymorphism of the renin-angiotensin aldosterone system.

Gene	Gene Polymorphism		Genotype Frequency		p
			TT+TG	GG	
AT1	-713T>G	POAG (n=165)	158 (95.8%)	7 (4.2%)	0.04
		NTG (n=208)	208 (100%)	0 (0.0%)	
		Control (n=198)	197 (99.5%)	1 (0.5%)	
			CC	CA+AA	
AT2	3123C>A (Female)	POAG (n=79)	34 (43.0%)	45 (56.0%)	
		NTG (n=120)	35 (29.2%)	85 (70.8%)	0.011
		Control (n=111)	54 (45.0%)	66 (55.0%)	

Association between two promoter polymorphisms in AT1R in POAG patients

A frequency of POAG carriers with combined homozygous -521T and homozygous -713G (4.2%) was significantly higher (p=0.011) than that of normals (0%) (**Table 8-1**). Only POAG patients, neither NTG nor normal subjects, had this genotype.

Table 8-1. Distribution of genotypes of AT1R -521T allele and -713G allele

Group	A	B	p
POAG (n=165)	7 (4.2%)	158 (95.8%)	0.011
NTG (n=208)	0 (0.0%)	208 (100.0%)	
Control (N=198)	0 (0.0%)	198 (100.0%)	

A: Subjects with two -521 alleles and two -713G alleles

B: Subjects not satisfying the criteria for Group A.

These results indicated that gene polymorphism of the renin-angiotensin aldosterone system is one of important genetic risk factors for development of glaucoma. Detection of AT1R/-731T>G polymorphisms makes possible the early diagnosis and early treatment of POAG. Especially, specific genotype of combined homozygous -521T and homozygous -713G in the AT1R gene is useful for the early diagnosis of POAG. Detection of the AT2R/3123C>A

polymorphisms make possible the early diagnosis and early treatment of female patient with NTG.

Clinical Characteristics of NTG Patients with AT2R 3123C>A Polymorphism and ACE I/D Polymorphism

The clinical features recorded in the glaucoma patients were age at diagnosis, untreated maximum IOP (defined as IOP at diagnosis), and visual field defects at the initial examination (defined as visual field defects at diagnosis. The severity of the visual field defects was scored from 1 to 5. Data obtained with different perimeters were combined using a five-point scale defined as follows: 1 = no alteration; 2 = early defect; 3 = moderate defect; 4 = severe defect; and 5 = light perception only or no vision. Field defects were judged to be early, moderate, or severe according to Kozaki's classification based on the results of Goldmann perimetry or the classification used for the Humphrey field analyzer. The former classification is most widely used in Japan.

Significant association of the clinical characteristics of visual field score was detected between male glaucoma patients with AT2R genotype. Visual field score in male POAG patients with C genotype had worse than those with A genotype (P=0.04, **Table 8-2**). No significant association of the clinical characteristics (age, IOP, and visual field score) was detected between female glaucoma

patients with C/C and those with C/A+A/A genotypes. The visual field score had a tendency to be worse in NTG patients with C/C genotype than those with C/A+A/A genotypes (P = 0.165).

However, when combined with ACE insertion/deletion polymorphism, female patients with NTG who carried C/C in the AT2R gene as well as ID+DD in the ACE gene had significantly worse visual field scores than the other three combined genotypes (P = 0.012; **Table 8-3, Figure 1**).

Table 8-2 Comparison of Clinical characteristics of male glaucoma patients according to AT2R genotypes

AT2 3123G>A
Male

Phenotype	Phenotype Variable	C	A	*P* value*
POAG	Age at diagnosis (ys)	57.0±10.9 (n=62)	56.9±14.0 (n=46)	0.808
	IOP at diagnosis (mm Hg)	26.8±6.7 (n=55))	27.5±6.7 (n=43)	0.522
	Visual field score at diagnosis	3.27±0.96 (n=62)	2.89±0.74 (n=46)	0.015

* P value for logistic regression analysis

Table 8-3 Comparison of clinical characteristcs of female patients with NTG according to ACE genotypes (Insertion/deletion) and AT2R genotypes (3123C>A)

Clinical characteristcs	ACE	I/I		I/D+D/D		
	AT2R	C/C	C/A + A/A	C/C	C/A + A/A	*P*
Age at diagnosis (ys)		63.6±10.9 (n=15)	57.0±11.2 (n=47)	56.2±14.1 (n=23)	58.5±12.0 (n=51)	0.313
IOP at diagnosis (mm Hg)		16.0±2.2 (n=16)	16.5±2.6 (n=43)	16.1±2.7 (n=20)	16.5±2.2 (n=49)	0.75
Visual field score at diagnosis		2.47±0.51 (n=17)	2.64±0.53 (n=47)	3.13±0.76 (n=23)	2.65±0.59 (n=52)	0.012†

* *P* value tested by Kruskal-Wallis test

† P<0.05

Partial nucleotide sequences of AT1R and AT2R genes containing the targeted polymorphism are as follows:

AT1R -713(the underlined "t") T>G

1861 attactgtaa actacagtca ccctactcac ctatctaaca ttaattgatt tttggtaaac

1921 taatctaatc ttgctttctg gcatcaacct cacttgacca tggtgtatag tccctttcat

1981 atgttattgg atTcaatttg cctacatttt gttgagaatt tttatctata ctcttaagaa

2041 atattgatct gtagtctcgt gatgtcttta tctggttttg ttatcagggt gatactggcc

2101 tcatagcatg agttgggaga tcatccttac tcttctattt tttggaagag tttgtgaaga

2161 attgatatta tttcttcttt aaatatttat tggggttttta aaatacattt ttaaaatgca

AT2R 3123(the underlined "c") C>A, the underlined oligonucleotide sequences were used for primers

ggattcagatttctctttgaaacatgcttgtgtttcttagtggggttttatatccatttttatcaggatt

tcctcttgaaccagaaccagtctttcaactcattgcatcattacaagacaacattgtaagagagatgag

cacttctaagttgagtatattataatagattagtactggattattcaggctttaggcatatgcttcttta

aaaacgctataaattatattcctcttgcatttcacttgagtggaggtttatagttaatctataactacat

attgaatagggctaggaatatagattaaatcatactcctatgc

(Based on GenBank accession No. AY536522, the AT2R 3123 corresponds nucleotide number 4926)

4741 gtgtttctta gtggggtttt atatccattt ttatcaggat ttcctcttga accagaacca

4801 gtctttcaac tcattgcatc atttacaaga caacattgta agagagatga gcacttctaa

4861 gttgagtata ttataataga ttagtactgg attattcagg ctttaggcat atgcttcttt

4921 aaaaacgcta taaattatat tcctcttgca tttcacttga gtggaggttt atagttaatc

4981 tataactaca tattgaatag ggctaggaat atagattaaa tcatactcct atgctttagc

5041 ttatttttac agttatagaa agcaagatgt actataacat agaattgcaa tctataatat

5101 ttgtgtgttc actaaactct gaataagcac tttttaaaaa actttctact cattttaatg

Example 4 Gene polymorphisms of the Endothelin gene associate with risk for developing normal-tension glaucoma

Methods

Patients

A total of 605 blood samples were collected. There were 178 POAG patients, 214 NTG patients, and 213 normal controls, and none of the subjects was related to others in this study. Patients were considered to have POAG if they had a normal open-angle, a cup-disc ratio greater than 0.7 with typical glaucomatous visual field loss on either Goldmann or Humphrey perimetry, and the absence of ocular, rhinologic, neurological or systemic disorders which might be responsible for the optic nerve damage. Patients with NTG had an IOP of 21 mmHg or lower. Patients with exfoliative glaucoma, pigmentary glaucoma, and corticosteroid-induced glaucoma were excluded. Control samples were obtained from Japanese subjects who had no known eye abnormalities except for cataracts. These subjects had IOPs below 21 mm Hg, had normal optic discs, and no family history of glaucoma.

Detection of G/T polymorphism of endothelin (ET) gene by Invader assay

DNA was isolated from peripheral blood lymphocytes by standard methods of phenol-chloroform extraction, and

G/T polymorphism (Lys/lys, Lys/Asn and Asn/Asn) at codon 198 in exon 5 of ET gene was determined by the Invader® assay. The primary probes (wild and mutant probes) and Invader® oligonucleotides (Invader® probe) used to detect the G/T polymorphism of ET gene are shown in **Table 9**.

Table 9

Mutation	nucleotide change	Target	Probe	Sequence	Tm	Dye
EDN Ex5 GT	G to T	Sense	Wild	Flap sequence-CTTGCCTTTCAGCTTGG	64.6	FAM
			Mutant	Flap-sequence-ATTGCCTTTCAGCTTGG	64.0	RED
			Invader	GTTGTGGGTCACATAACGCTCTCTGGAGGGT	76.9	

Invader® assay FRET-detection 96-well plates (Third Wave Technologies, Inc, Madison, WI) contains the generic components of an Invader® assay (Cleavase® enzyme VIII, FRET probes, MOPS buffer, and polyethylene glycol) dried in each of the individual wells. In brief, 8 µl of the primary probe/Invader®/mixture and total DNA (10 ng) samples were added to each well of a 96-well plate, and were denatured by incubation at 95° C for 10 min. After 15 µl of mineral oil (Sigma, St. Louis, MO) was overlaid on all reaction wells, the plate was incubated isothermally at 63° C for 2 hours in a PTC-100 thermal cycler (MJ Research, Waltham, MA) and then kept at 4° C until fluorescence measurements. The fluorescence intensities were measured on a CytoFlour 4000 fluorescence plate reader (Applied Biosystems, Foster City, CA) with excitation at 485 nm/20 nm (wavelength/ bandwidth) and emission at 530 nm/25 nm for FAM dye; excitation at 560 nm/20 nm and emission at 620 nm/40 nm for Redmond RED (RED) dye. Each sample was tested in duplicate in the same plate and two fluorescence measurements were performed in each plate. Thus, four measurements were obtained for each sample and they were averaged.

Results

The genotype frequencies of G/T polymorphism (Lys/lys, Lys/Asn and Asn/Asn) at codon 198 in exon 5 of ET gene are

presented in Table 10.

Table 10. The genotype frequency at codon 198 in exon 5 of ET gene

Group	n	Genotype Frequency			p	Genotype Frequency		p
		Lys/lys	Lys/Asn	Asn/Asn		Lys/lys	Lys/Asn + Asn/Asn	
Control	213	94 (44.1%)	93 (43.7%)	26 (12.2%)		94 (44.1%)	119 (55.9%)	
NTG	214	120 (56.1%)	72 (33.6%)	22 (10.3%)	0.046	120 (56.1%)	94 (43.9%)	0.014
POAG	178	82 (46.1%)	77 (43.3%)	19 (10.7%)		82 (46.1%)	96 (53.9%)	

These results indicated that Lys/Lys homozygote of ET-1 gene at codon 198 in exon 5 is one of the risk factor to develop or progress the NTG, and detection of the Lys/Lys homozygote makes possible the early diagnosis and early treatment of NTG.

Partial sequence of EDN1 comprising codon 198 is as follows:

EDN1 Codon 198 (underlined): aag (Lys) to aat (Asn)

9061 ttgaggtttt atcaaagagt tgcggcgggt ggtgaaagtt cacaaccaga ttcaggtttt

9121 gtttgtgcca gattctaatt ttacatgttt cttttgccaa agggtgattt ttttaaaata

9181 acatttgttt tctcttatct tgctttatta ggtcggagac catgagaaac agcgtcaaat

9241 catcttttca tgatcccaag ctgaaaggca agccctccag agagcgttat gtgacccaca

9301 accgagcaca ttggtgacag accttcgggg cctgtctgaa gccatagcct ccacggagag

9361 ccctgtggcc gactctgcac tctccaccct ggctgggatc agagcaggag catcctctgc

(tga is the translation termination codon)

Example 5 Novel *MYOC* Gene Mutation, Phe369Leu, in Japanese Patients with Primary Open-angle Glaucoma Detected by

Denaturing High-performance Liquid Chromatography

Purpose: To screen for mutations in the *MYOC* gene in Japanese patients with primary open-angle glaucoma (POAG) using denaturing high-performance liquid chromatography (DHPLC).

Materials and Methods

Patients

Blood samples were collected from 171 POAG patients and 100 normal subjects at seven Japanese medical institutions. The subjects were unrelated, and their mean age at the time of examination was 55.1 ± 16.0 (± standard deviation) years for the patients with POAG and 70.5 ± 10.6 years for the normal subjects. We purposely selected older control subjects to reduce the probability that a subset of them would develop glaucoma.

A detailed family history was obtained by interviews in 55 POAG patients (32.2%). There were 91 men (53.2%) in the POAG patients, and 41 men (41.0%) in the normal subjects.

DNA Extraction and PCR Conditions

Genomic DNA was isolated from peripheral blood lymphocytes by standard methods. The seven exonic regions of the *MYOC* gene were amplified by polymerase chain reaction (PCR) using the primer sets listed in **Table 11.**

For high-throughput analysis of the patients, samples from three patients were pooled. The PCR reaction was performed with a thermal cycler (iCycler; Bio Rad, Hercules, CA) in a total volume of 25 µl. The PCR conditions were: denaturation at 95° C for 9 min; followed by 35 cycles at 95° C for 1 min; 58° C for 30 sec (Table 1); and 72° C for 1.5 min; a final extension step was then carried out at 72° C for 7 min. For heteroduplex formation, each PCR product (25 µl) was denatured at 95° C for 5 min and gradually cooled to 25° C.

For analyses of a few samples, each of seven exonic regions was amplified simultaneously by PCR in a 96-well plate (96-well Multiplate, MLP-9601; MJ Research, Waltham, MA). Seven wells were used for each patient. Primer sets were designed to be effective using a single annealing temperature of 58° C (**Table 11**).

Table 11. Primer sequences, product size, and PCR annealing and DHPLC analysis temperatures

Exon		Primer sequences (5' to 3')	Product size (bp)	PCR Tm (°C)	DHPLC Tm (°C)
1A	F	AGC ACA GCA GAG CTT TCC AGA GGA	302	58.0	61.9
	R	CTC CAG GTC TAA GCG TTG G			
1B	F	CAG GCC ATG TCA GTC ATC CA	298	58.0	61.2, 64.5
	R	TCT CAT TTT CTT GCC TTA GTC			
1C	F	GAA ACC CAA ACC AGA GAG	255	58.0	61.0, 63.5
	R	ATA TCA CCT GCT GAA CTC AGA GTC			
2A	F	CCT CAA CAT AGT CAA TCC TTG GGC	245	58.0	56.3, 59.3
	R	ACA TGA ATA AAG ACC ATG TGG GCA			
3A	F	GAT TAT GGA TTA AGT GGT GCT TCG	375	58.0	59.3, 61.3, 62.3
	R	TGT CTC GGT ATT CAG CTC AT			
3B	F	CAT ACT GCC TAG GCC ACT GGA	337	58.0	60.9, 61.4
	R	ATT GGC GAC TGA CTG CTT AC			
3C	F	GAA TCT GGA ACT CGA ACA AA	333	58.0	59.7, 61.7
	R	CTG AGC ATC TCC TTC TGC CAT			

Denaturing HPLC Analysis

For high-throughput analysis, a 25 µl volume of PCR products from the three patients was automatically injected into the chromatograph for analysis using the WAVE® System for DHPLC analysis (Transgenomic, Omaha, NE). The DHPLC melting temperatures are listed in Table 1. For analysis of a small number of samples, following 96-well-plate PCR, the plate was next placed in a WAVE® System programmed to automatically analyze each well at two to three melting temperatures. Approximately 3 hrs was sufficient time to analyze one individual's sample.

When abnormal chromatographic patterns were detected in the pooled samples by the high-throughput protocol, the sample was reanalyzed individually in the WAVE® System. The PCR product that showed the abnormal chromatographic

pattern was then sequenced.

Direct DNA Sequencing

For direct sequencing, PCR products were purified with a QIA Quick PCR purification kit (Qiagen, Valencia, CA) to remove unused primers and precursors. The PCR products were directly sequenced with the same forward and reverse PCR amplification primers on an ABI310 automated sequencer using BigDye chemistry according to the manufacturer's recommended protocol (Applied Biosystems, Foster City, CA).

Results

Screening of Pools of DNA in 171 Patients

Four DHPLC tracing patterns in the Exon3C region were shown in **Figure 2**. The upper most pattern (**A**) has a normal appearance, while the middle pattern (**B**) showed a broad shoulder, and the lower patterns (**C** and **D**) had a characteristic double peak pattern indicative of sequence variations in this region. Sequencing analysis of samples B, C, and D revealed Thr448Pro, Pro481Ser, and Ala488Ala mutations (**Table 12**).

Four glaucoma-causing mutations were identified in 5 (2.9%) of 171 patients with POAG. In addition, eight polymorphisms and five synonymous codon changes were identified (**Table 12**). One novel missense mutation, Phe369Leu detected in exon 3 (**Figure 3**) was not present in

100 normal Japanese subjects. The three other missense mutations, Ile360Asn, Ala363Thr, and Thr448Pro have been reported in Japanese patients with POAG.

Table 12. MYOC mutations and polymorphisms in patients with POAG and controls

	Exon	Sequence change	Amino acid change	Frequency patients	Frequency controls
Mutations	3	c.1079T>A	Ile360Asn	1/171	0/100
	3	c.1087G>A	Ala363Thr	2/171	0/100
	3	c.1105T>C	Phe369Leu*	1/171	0/100
	3	c.1342A>C	Thr448Pro	1/171	0/100
Polymorphisms	1	c.34G>C	Gly12Arg	1/171	2/100
	1	c.57G>T	Gln19His	1/171	1/100
	1	c.136C>T	Arg46Stop	1/171	1/100
	1	c.210C>T	Val70Val†	2/171	0/100
	1	c.227G>A	Arg76Lys	14/171	9/100
	1	c.369C>T	Thr123Thr	1/171	0/100
	1	c.473G>A	Arg158Gln	1/171	1/100
	2	c.611C>T	Thr204Met	0/171	1/100
	2	c.624C>G	Asp208Glu	5/171	2/100
	3	c.864C>T	Ile288Ile	1/171	0/100
	3	c.1110G>A	Pro370Pro	0/171	1/100
	3	c.1441C>T	Pro481Ser	1/171	0/100
	3	c.1464C>T	Ala488Ala	3/171	1/100

* Novel myocilin mutation; † novel myocilin polymorphism.

Screening of Individual Patients by Plate PCR followed by DHPLC

A DHPLC tracing from a patient with POAG is shown in Figure 4. In the exon3B region, an abnormal tracing indicative of sequence variation can be seen, which proved to represent a Phe369Leu mutation on direct sequencing.

Partial nucleotide sequences for MYOC exon 3 gene containing the targeted polymorphism is as follows:

MYOC Exon 3, codon 369(underlined) TTC(Phe) to CTC(Leu)

301 actggaaagc acgggtgctg tggtgtactc ggggagcctc tatttccagg gcgctgagtc

361 cagaactgtc ataagatatg agctgaatac cgagacagtg aaggctgaga aggaaatccc

421 tggagctggc taccacggac agttcccgta ttcttggggt ggctacacgg acattgactt

481 ggctgtggat gaagcaggcc tctgggtcat ttacagcacc gatgaggcca aaggtgccat

541 tgtcctctcc aaactgaacc cagagaatct ggaactcgaa caaacctggg agacaaacat

The nucleotide sequences of MYOC exon 1-3 are available from GenBank, Accession Nos. AB006686-AB006688

Example 6 Variants in Optineurin Gene and their Association with Tumor Necrosis Factor-α Polymorphisms in Japanese Patients with Glaucoma

Purpose: To investigate sequence variations in the optineurin (OPTN) gene and their association with TNF-α polymorphism in Japanese patients with glaucoma.

SUBJECTS AND METHODS

Patients and Control Subjects

A total of 629 blood samples were collected at seven institutions in Japan. There were 194 POAG patients, 217 NTG patients, and 218 normal controls, and none of the subjects was related to others in this study. The patients whose age at diagnosis was less than 35 years and patients with over −5.5 D of myopia were excluded. POAG patients with *MYOC* mutations were also excluded.

DNA Extraction and PCR Conditions

Genomic DNA was isolated from peripheral blood lymphocytes by phenol-chloroform extraction. The 13 exonic coding regions of the *OPTN* gene were amplified by polymerase chain reaction (PCR) using the primer sets listed in **Table 13**. A 20-base GC-clamp was attached to some of the forward primers to detect mutations in the higher melting temperature domain by DHPLC analysis (Narayanaswami G et. al., Genet Test. 2001;5:9-16). In high-throughput analysis, samples from three patients were pooled. PCR was performed with a thermal cycler (iCycler, Bio-Rad; Hercules, CA) in a total volume of 20 µl containing; 45 ng of genomic DNA, 2 µl GeneAmp 10x PCR buffer II, 2 µl of GeneAmp dNTP mix with a 2.0 mM concentration of each dNTP, 2.4 µl of a 25 mM $MgCl_2$ solution; 4 pmol of each primer, and 0.1 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA). PCR conditions were; denaturation at 95° C for 9 min, followed by 35 cycles at 95° C for 1 min, 55° to 60° C for 30 sec (**Table 13**), and 72° C for 1 min and 30 sec, and a final extension step at 72° C for 7 min.

Table 13. Primer sequences, PCR product sizes, and PCR annealing and DHPLC analysis temperatures

Exon		Primer Sequences (5' to 3')	PCR product size (bp)	PCR Tm (°C)	DHPLC Tm (°C)
4	F	CCAGTGGGTTTGTGGGACTCC	317	60	61.7
	R	AAAGGGATGGCATTTCTTGCA			
5	F	GTCCACTTTCCTGGTGTGTGACT	277	55	58.7
	R	CAACATCACAATGGATCG			
6	F	AGCCTTAGTTTGATCTGTTCATTCA	293	60	57.0, 62.5
	R	GTTTCATCTTTCCAGGGGAGGCT			
7	F	GC-clamp AATCCCTTGCATTTCTGTTTTT	188	55	59.4, 61.4, 62.4
	R	GTGACAAGCACCCAGTGACGA			
8	F	GC-clamp GGTTACTCTCTTCTTAGTCTTTGGA	320	57	54.6, 58.5
	R	GGGTGAACTGTATGGTATCTTAATT			
9	F	GC-clamp GCTATTTCTCTTAAAGCCAAAGAGA	242	55	57.4, 59.4
	R	CAGTGGCTGGACTACTCTCGT			
10	F	GC-clamp GTCAGATGATAATTGTACAGATAT	227	55	57.8, 59.8
	R	AATGTATATTTCAAAGGAGGATAAA			
11	F	CCACTGCGACGTAAAGGAGCA	286	60	57.5, 59.5
	R	CAAATCCGAATTCCAATCTGTATAA			
12	F	GC-clamp GGTTGGGAGGCAAGACTATAAGTT	233	60	55.5, 56.5
	R	TTCTGTTCATTACTAGGCTATGGAA			
13	F	CAGGCAGAATTATTTCAAAACCAT	264	60	58.9, 61.9
	R	CGAGAATACAGTCAGGGCTGG			
14	F	GCACTACCTCCTCATCGCATAAACA	260	60	56.7, 59.7
	R	GGCCATGCTGATGTGAGCTCT			
15	F	GC-clamp GGACTGTCTGCTCAGTGTTGTCA	282	60	56.0, 59.0, 61.0
	R	GGTGCCTTGATTTGGAATCCA			
16	F	GC-clamp CACAACTGCCTGCAAAATGGAACT	294	60	61.7
	R	GAGGCAAAATATTTGAGTGAAAACA			

GC-clamp: CGCCCGCCGCCGCCCGCCGC

Denaturing HPLC Analysis

DHPLC analysis was performed using the WAVE® SYSTEMS (Transgenomic, Omaha, NE). For heteroduplex formation, products of each PCR (20 µl) were denatured at 95° C for 5 min and gradually cooled to 25° C. The annealed PCR products from the three mixed samples were automatically injected into a DNASep® cartridge (Transgenomic, Omaha, NE).

Buffer A (Transgenomic, Omaha, NE) was made up of

0.1 M triethylammonium acetate (TEAA), and Buffer B of 0.1 M TEAA and 25% acetonitrile. Analysis was carried out at a flow rate of 0.9 ml/min and the Buffer B gradient increased by 2%/min for 4.5 min. Elution of DNA fragments from the cartridge was detected by absorbance at 260 nm. The temperatures used for the analysis were selected according to the sequences of the DNA fragments. The WAVEMAKER software (v.4.1, Transgenomic, Omaha, NE) predicted the melting behavior of the DNA fragments at various temperatures. The predicted melting domains within the DNA fragment determined the temperatures for the DHPLC analysis (**Table 13**). When abnormal chromatographic patterns were detected in a pool of three samples, each of the three samples was re-analyzed individually in the WAVE® SYSTEM. Then, the PCR product that showed an abnormal chromatographic pattern was sequenced. Once a correlation between abnormal chromatographic patterns and base changes was confirmed by direct sequencing analysis, additional sequencing analyses were not performed when any of the known abnormal chromatographic patterns were observed in the DHPLC analysis.

Direct DNA Sequencing

To detect mutations by direct sequencing, the PCR products were first purified with the QIAquick PCR Purification Kit (QIAGEN, Valenica, CA, USA) to remove

unreacted primers and precursors. The sequencing reactions were then performed using the ABI PRISM BigDye Terminator (v.3.1) Cycle Sequencing Kit, according to the manufacturer's protocol (Applied Biosystems). The data were collected by the ABI PRISM 310 Genetic Analyzer and analyzed by the ABI PRISM sequencing analysis program (v.3.7).

Genotyping OPTN c.412G>A (Thr34Thr) Polymorphism

The G to A substitution at position c.412 in exon 4 of the *OPTN* gene was detected by using restriction enzyme, *Hpy*CH_4IV (New England BioLabs, Beverly, MA), with the same primers listed in **Table 13** for the DHPLC analysis. The G allele sequence was cut into two fragments (188 bp + 129 bp) by *Hpy*CH_4IV, while the A allele sequence remained intact (317 bp). The polymorphism was confirmed by restriction-enzyme assay and the chromatographic pattern of DHPLC.

Genotyping OPTN c.603T>A (Met98Lys) Polymorphism

The T to A substitution at position c.603 in exon 5 of the *OPTN* gene was detected by restriction enzyme, *Stu* I (TaKaRa, Shiga, Japan), using the same primers as for the DHPLC analysis (Table 13). The A allele sequence was cut into two fragments (175 bp + 102 bp) by *Stu* I, while the T allele sequence remained intact (277 bp). The polymorphism was confirmed by restriction-enzyme assay and the

chromatographic pattern of DHPLC.

Genotyping OPTN c.1944G>A (Arg545Gln) Polymorphism

The G to A substitution at position c.1944 in exon 16 of the *OPTN* gene was analyzed by the Invader assay provided by the Research Department of R&D Center, BML (Saitama, Japan). The polymorphism was confirmed by Invader® assay and by the chromatographic pattern of DHPLC.

Genotyping TNF-α -308G>A Polymorphism

Genotyping the -308G>A polymorphism in the TNF-α promoter region was performed by using restriction enzyme *Nco*I (New England BioLabs, Beverly, MA), with the forward primer, 5'-AGGCAATAGGTTTTGAGGGCCAT-3', and the reverse primer, 5'-GTAGTGGGCCCTGCACCTTCT -3'. The forward primer contained one nucleotide mismatch (bold and underlined), which allowed the use of the restriction enzyme. The G allele sequence was cut into two fragments (192 bp +20 bp) by *Nco*I while the A allele sequence remained intact (212 bp).

Genotyping TNF-α -857C>T Polymorphism

Genotyping the -857C>T polymorphism in the TNF-α promoter region was performed by using restriction enzyme *Hinc*II (TaKaRa, Shiga, Japan), with the forward primer, 5'-AAGTCGAGTATGGGGACCCCCCGTTAA-3', and the reverse primer, 5'-CCCCAGTGTGTGGCCATATCTTCTT-3'. The forward primer contained one nucleotide mismatch (bold and underlined), which

allowed the use of the restriction enzyme. The C allele sequence was cut into two fragments (106 bp +25 bp) by *Hinc*II, while the T allele sequence remained intact (131 bp). Transcriptional activity of the -857T allele was significantly greater than that of -857C allele.

Genotyping TNF-α -863C>A Polymorphism

Genotyping the -863C>A polymorphism in the TNF-α promoter region was done by using restriction enzyme *EcoNI* (New England BioLabs, Beverly, MA) with the forward primer, 5'-GCTGAGAAGATGAAGGAAAAGTC-3', and the reverse primer, 5'-CCTCTACATGGCCCTGTCCT-3'. The reverse primer contained one nucleotide mismatch (bold and underlined), which allowed the use of the restriction enzyme. The C allele sequence was cut into two fragments (183 bp +23 bp) by *EcoNI*, while the A allele sequence remained intact (206 bp). Transcriptional activity of the -863A allele was significantly greater than that of -863C allele.

Statistical Analyses

The frequencies of the genotypes and alleles in patients and controls were compared with the chi-square test and Fisher's exact test. The odds ratio and 95% confidence intervals (CI) also were calculated. The Hardy-Weinberg equilibrium for the observed frequencies was also calculated. Comparisons of the clinical characteristics between the two groups were performed using Mann-Whitney *U*

test or Student's unpaired *t*-test when appropriate. Logarithmic transformation was performed on skewed distribution clinical data which were the IOP at diagnosis of POAG, visual field score at diagnosis of NTG, and POAG to obtain a normal distribution for performing analysis of variance (ANOVA). One-way ANOVA was used to compare three clinical characteristics among patients with 4 different combinations of the TNF-α/-857C>T and optineurin/412G>A genotypes, or the TNF-α/-863C>A and optineurin/603T>A genotypes (see **Table 17**).

Statistical analysis was performed with SPSS program (SPSS Inc., Chicago, USA). A *P* value of <0.05 was considered to be significant.

RESULTS

OPTN Variants in Japanese Subjects

A total of 629 Japanese subjects were studied, and the results are presented in **Table 14**.

Table 14. OPTN variants observed in glaucoma patients and control subjects

Location	Sequence Changes	Codon Changes	Frequency in Subjects (%) POAG	NTG	Control
Exon 4	c.386C>G	His26Asp	1 / 201 (0.5)	0 / 232 (0)	0 / 218 (0)
Exon 4	c.449-451delCTC	Leu47del	0 / 201 (0)	0 / 232 (0)	1 / 218 (0.5)
Exon 5	c.603T>A	Met98Lys	33 / 201 (16.4)	50 /232 (21.6)	36 / 218 (16.5)
Exon 16	c.1944G>A	Arg545Gln	14 / 192 (7.3)	15 / 222 (6.8)	11 / 214 (5.1)
Exon 4	c.412G>A	Thr34Thr	69 / 201 (34.3)	74 / 232 (31.9)	52 / 218 (23.9)
Exon 4	c.421G>A	Pro37Pro	0 / 201 (0)	1 / 232 (0.4)	0 / 218 (0)
Exon 4	c.457C>T	Thr49Thr	2 / 201 (1)	0 / 232 (0)	0 / 218 (0)
Exon 16	c.2023C>T	His571His	0 / 162 (0)	0 / 193 (0)	2 / 196 (1.0)
Intron 4	c.476+15C>A		0 / 201 (0)	0 / 232 (0)	1 / 218 (0.5)
Intron 6	c.863-10G>A *		N/C†	N/C	N/C
Intron 6	c.863-5C>T *		N/C	N/C	N/C
Intron 8	c.1089+20G>A		4 / 133 (3.0)	11 / 172 (6.4)	4 / 126 (3.2)
Intron 9	c.1192+19C>T		0 / 133 (0)	4 / 172 (2.3)	3 / 130 (2.3)
Intron 11	c.1458+28G>C		1 / 133 (0.8)	4 / 172 (2.3)	0 / 157 (0)
Intron 15	c.1922+10G>A		2 / 133 (1.5)	4 / 172 (2.3)	1 / 157 (0.6)
Intron 15	c.1922+12G>C		0 / 133 (0)	1 / 172 (0.6)	0 / 157 (0)
Intron 15	c.1923-48C>A *		N/C	N/C	N/C

* Sequence variation was found by direct sequencing analysis.
† Not checked

Seventeen sequence changes were identified in the glaucoma patients and control subjects. Among these, three were missense changes, one was a deletion of one amino acid residue, four were synonymous codon changes, and nine were changes in noncoding sequences. One possible disease causing-mutation, His26Asp, was identified in one POAG proband and was not present in the 218 normal Japanese controls. Her brother aged 55 harbored the mutation and was diagnosed as NTG. Her brother's daughter aged 23 also had the mutation and showed cupping of the optic nerve head

with a cup/disk ratio of 0.7 with no sign of visual field defect by Humphrey perimetry .

A deletion of Leu47 (3-bp deletion, CTC) was found in 1 control. A Met98Lys was identified in 33 POAG patients, 48 NTG patients, and 36 controls, and an Arg545Gln was identified in 11 POAG patients, 15 NTG patients, and 11 controls.

Four synonymous nucleotide substitutions, c.412G>A (Thr34Thr), c.421G>A (Pro37Pro), c.457C>T (Thr49Thr), and c.2023C>T (His571His) were found. The Thr34Thr substitution was present in 69 (35.6%) POAG patients, 69 (31.8%) NTG patients, and 52 (23.9%) controls, and the Pro37Pro was found in 1 NTG patient. The Thr49Thr was identified in 1 POAG patient, and the His571His was present in 2 controls.

Distribution of OPTN Variants in Japanese Subjects

The Thr34Thr (c.412G>A) polymorphism was significantly associated with POAG and NTG (**Table 15**). A significant association was found in patients with POAG (P =0.009 in genotype frequency: G/G vs G/A+A/A, and P = 0.003 in allele frequency). No significant difference was detected between glaucoma patients and controls in either genotype or allele frequency for the Met98Lys (c.603T>A) or the Arg545Gln (c.1944G>A) polymorphisms. However, the Met98Lys polymorphism had a higher tendency to be associated with NTG than with POAG. The observed genotype

frequencies were in agreement with those predicted by the Hardy-Weinberg equilibrium.

Table 15. Genotype distribution and allele frequency of optineurin gene polymorphisms in glaucoma patients and controls c. 412G>A (Thr34Thr)

Phenotype	n	Genotype frequency (%) G/G	G/A	A/A	P value*	Genotype frequency (%) G/G	G/A+A/A	P value*	Genotype frequency (%) G/G+G/A	A/A	P value†	Allele frequency (%) G	A	P value*
POAG	194	125 (64.4)	61 (31.4)	8 (4.1)	0.011 ‡	125 (64.4)	69 (35.6)	0.008 §	186 (95.8)	8 (4.1)	0.051	311 (80.2)	77 (19.8)	0.003 §
NTG	217	148 (68.2)	62 (28.6)	7 (3.2)	0.078	148 (68.2)	69 (31.8)	0.064	210 (96.8)	7 (3.2)	0.105	358 (82.5)	76 (17.5)	0.034 ‡
Control	218	166 (76.1)	50 (22.9)	2 (1.0)		166 (76.1)	52 (23.9)		216 (99.0)	2 (1.0)		382 (87.8)	54 (12.4)	
c.6037T>A (Met98Lys)														
Phenotype	n	Genotype frequency (%) T/T	T/A	A/A	P value*	Genotype frequency (%) T/T	T/A+A/A	P value*	Genotype frequency (%) T/T+T/A	A/A	P value†	Allele frequency (%) T	A	P value*
POAG	194	161 (83.0)	32 (16.5)	1 (0.5)	0.990	161 (83.0)	33 (17.0)	0.893	193 (99.5)	1 (0.5)	1	354 (91.2)	34 (8.8)	0.888
NTG	217	169 (77.9)	43 (19.8)	5 (2.3)	0.133	169 (77.9)	48 (22.1)	0.139	212 (97.7)	5 (2.3)	0.122	381 (87.9)	53 (12.2)	0.071
Control	218	182 (83.5)	35 (16.0)	1 (0.5)		182 (83.5)	36 (16.5)		217 (99.6)	1 (0.5)		399 (91.5)	37 (8.5)	

* *P* value for $\chi 2$ test.
† *P* value for Fisher's exact test.
‡ $P<0.05$
§ $P<0.01$

Three clinical characteristics of the glaucoma patients, viz., age at diagnosis, IOP at diagnosis, and

visual field score at diagnosis, were examined for association with c.412G>A (Thr34Thr) or c.603T>A (Met98Lys) polymorphisms (**Table 16**). The glaucoma patients did not show an association with the clinical characteristics with the c.412G>A polymorphism. POAG patients with the G/A+A/A genotype (or 412A carriers) tended to have more advanced visual field scores than those with the G/G genotype (or non-412A carriers; $P = 0.093$). POAG patients with the 603T>A polymorphism showed a weak association with age at diagnosis ($P = 0.046$).

Table16 Comparison of clinical characteristcs of glaucoma patients according to *OPTN* genotypes

c.412G>A (Thr34Thr)

	Phenotype Variable	G/G		G/A+A/A		*P* value*
POAG	Age at diagnosis (ys)	58.1 ± 11.8	(n = 123)	58.8 ± 12.6	(n = 69)	0.663
	IOP at diagnosis (mm Hg)	27.0 ± 6.5	(n = 112)	26.1 ± 5.0	(n = 60)	0.360
	Visual field score at diagnosis	3.0 ± 0.9	(n = 125)	3.2 ± 0.9	(n = 69)	0.093
NTG	Age at diagnosis (ys)	58.7 ± 11.7	(n = 148)	56.6 ± 11.2	(n = 69)	0.206
	IOP at diagnosis (mm Hg)	16.4 ± 2.6	(n = 139)	16.6 ± 2.2	(n = 67)	0.848
	Visual field score at diagnosis	2.8 ± 0.7	(n = 148)	2.7 ± 0.7	(n = 69)	0.135

c.603T>A (Met98Lys)

	Phenotype Variable	T/T		T/A+A/A		*P* value*
POAG	Age at diagnosis (ys)	57.6 ± 11.9	(n = 159)	62.2 ± 12.4	(n = 33)	0.046†
	IOP at diagnosis (mm Hg)	26.8 ± 5.8	(n = 143)	26.5 ± 7.1	(n = 29)	0.931
	Visual field score at diagnosis	3.1 ± 0.9	(n = 161)	3.2 ± 0.9	(n = 33)	0.280
NTG	Age at diagnosis (ys)	58.4 ± 11.6	(n = 169)	56.6 ± 11.6	(n = 48)	0.304
	IOP at diagnosis (mm Hg)	16.4 ± 2.4	(n = 160)	16.8 ± 2.6	(n = 46)	0.270
	Visual field score at diagnosis	2.8 ± 0.7	(n = 169)	2.8 ± 0.6	(n = 48)	0.318

* *P* values for Mann-Whitney U test.
† $P<0.05$

Association between OPTN Polymorphism and TNF-α Polymorphism in Glaucoma Patients

No significant difference in genotype or allele frequency was noted between patients and controls for the three polymorphisms of the -308G>A, -857C>T or -863C>A. In addition, the glaucoma patients did not show an association with the clinical characteristics for the three polymorphisms (data not shown). The observed genotype frequencies were in agreement with those predicted by the Hardy-Weinberg equilibrium.

However, among individuals with the C/T+T/T genotype (or -857T carriers) in the TNF-α gene, 44.1 % of POAG patients were G/A+A/A genotypes (or 412A carriers) in the *OPTN* gene compared to 21.6 % of controls (**Table 17**). This difference in frequency was significant (P = 0.006). Among individuals with the C/A+A/A genotype (or -863A carriers) in the TNF-α gene, 603A carriers (or Lys98 carriers) in the *OPTN* gene were significantly associated with POAG as well as NTG (P = 0.008 and 0.027, respectively).

Table 17 Distribution of optineurin genotypes (c.412G>A and c.603T>A) according to TNF-α genotypes (-857C>T and -863C>A)

c.412G>A (Thr34Thr)

Phenotype	-857C>T c.412G>A	C/C (%) G/G	G/A + A/A	*P* value*	Odds ratio 95 % CI	C/T+T/T (%) G/G	G/A + A/A	*P* value*	Odds ratio 95 % CI
POAG		92 (68.1)	43 (31.9)	0.204	1.40 (0.83-2.37)	33 (55.9)	26 (44.1)	0.006‡	2.86 (1.34-6.08)
NTG		97 (65.5)	51 (34.5)	0.077	1.58 (0.95-2.62)	51 (73.9)	18 (26.1)	0.531	1.28 (0.59-2.77)
Control		108 (75.0)	36 (25.0)			58 (78.4)	16 (21.6)		

Phenotype	-863C>A c.412G>A	C/C (%) G/G	G/A + A/A	*P* value*	Odds ratio 95 % CI	C/A+A/A(%) G/G	G/A + A/A	*P* value*	Odds ratio 95 % CI
POAG		91 (64.5)	50 (35.5)	0.017	1.84 (1.11-3.05)	34 (64.2)	19 (35.8)	0.280	1.56 (0.69-3.53)
NTG		110 (69.2)	49 (30.8)	0.114	1.49 (0.91-2.46)	38 (65.5)	20 (34.5)	0.341	1.47 (0.66-3.28)
Control		124 (77.0)	37 (23.0)			42 (73.7)	15 (26.3)		

c.603T>A (Met98Lys)

Phenotype	-857C>T c.603T>A	C/C (%) T/T	T/A + A/A	*P* value*	Odds ratio 95 % CI	C/T+T/T (%) T/T	T/A + A/A	*P* value*	Odds ratio 95 % CI
POAG		112 (83.0)	23 (17.0)	0.811	1.08 (0.57-2.03)	49 (83.1)	10 (16.9)	0.925	0.96 (0.39-2.37)
NTG		111 (75.0)	37 (25.0)	0.056	1.75 (0.98-3.13)	58 (84.1)	11 (15.9)	0.795	0.89 (0.37-2.14)
Control		121 (84.0)	23 (16.0)			61 (82.4)	13 (17.6)		

Phenotype	-863C>A c.603T>A	C/C (%) T/T	T/A + A/A	*P* value*	Odds ratio 95 % CI	C/A+A/A (%) T/T	T/A + A/A	*P* value*	Odds ratio 95 % CI
POAG		123 (87.2)	18 (12.8)	0.127	0.61 (0.33-1.15)	38 (71.7)	15 (28.3)	0.008‡	4.11 (1.37-12.27)
NTG		125 (78.6)	34 (21.4)	0.636	1.14 (0.66-1.97)	44 (75.9)	14 (24.1)	0.027†	3.31 (1.10-9.91)
Control		130 (80.7)	31 (19.3)			52 (91.2)	5 (8.8)		

* *P* values for $\chi 2$ test.
† $P<0.05$
‡ $P<0.01$

The clinical characteristics of these combined genotypes, such as age at diagnosis, IOP at diagnosis, and visual field score at diagnosis are shown in **Table 18**. The POAG patients who were TNF-α/-857T and optineurin/412A carriers had significantly worse ($P = 0.020$) visual field scores than those who were TNF-α/-857T and non-optineurin/412A carriers. However, there was no significant difference in the three clinical features of POAG patients among the four genotypes of combined -857T>A and c.412G>A

polymorphisms (Table 6) by one-way ANOVA: $P = 0.823$ for age at diagnosis; $P = 0.692$ for IOP at diagnosis; and $P = 0.152$ for visual field score at diagnosis.

POAG patients who were TNF-α/-863A and optineurin/603A carriers had significantly worse ($P = 0.026$) visual field scores than those who were TNF-α/-863A and non- optineurin/603A carriers. However, there was no significant difference in the visual field score of POAG patients among the four genotypes of combined -863C>A and -603T>A polymorphisms (Table 6, one-way ANOVA: $P = 0.200$).

Table 18 Comparison of clinical characteristics of glaucoma patients according to TNF-α genotypes (-857T and -863A) and optineurin genotypes (412A and 603A)

c.412G>A (Thr34Thr)				
	(TNF-α genotypes)	C/T+T/T (-857T carrier)		
	(*OPTN* genotypes)	G/G	G/A+A/A	*P* value*
POAG	Age at diagnosis (ys)	57.1 ± 10.7 (n = 32)	57.6 ± 13.1 (n = 26)	0.802
	IOP at diagnosis (mm Hg)	26.4 ± 6.1 (n = 30)	26.4 ± 5.5 (n = 20)	0.786
	Visual field score	2.9 ± 0.9 (n = 33)	3.3 ± 0.8 (n = 26)	0.020†
NTG	Age at diagnosis (ys)	58.4 ± 11.1 (n = 51)	59.3 ± 10.5 (n = 18)	0.790
	IOP at diagnosis (mm Hg)	16.4 ± 2.6 (n = 46)	16.1 ± 2.3 (n = 17)	0.520
	Visual field score	2.8 ± 0.8 (n = 51)	2.6 ± 0.5 (n = 18)	0.335
c.603T>A (Met98Lys)				
	(TNF-α genotypes)	C/A+A/A (-863A carrier)		
	(*OPTN* genotypes)	T/T	T/A+A/A	*P* value*
POAG	Age at diagnosis (ys)	56.3 ± 10.5 (n = 38)	62.0 ± 13.8 (n = 15)	0.074
	IOP at diagnosis (mm Hg)	27.9 ± 6.5 (n = 36)	26.9 ± 8.7 (n = 14)	0.488
	Visual field score	3.0 ± 0.8 (n = 38)	3.5 ± 0.9 (n = 15)	0.026†
NTG	Age at diagnosis (ys)	57.9 ± 11.4 (n = 44)	56.9 ± 11.9 (n = 14)	0.579
	IOP at diagnosis (mm Hg)	16.2 ± 2.4 (n = 40)	16.9 ± 2.4 (n = 14)	0.364
	Visual field score	2.9 ± 0.5 (n = 44)	2.7 ± 0.6 (n = 14)	0.296

* *P* values for Mann-Whitney U test.
† $P<0.05$

Partial nucleotide sequence of OPTN exon 4, comprising the targeted polymorphism, 412G>A (underlined)

caacagtgac ttttccacag gaacttctgc aatgtcccat caacctctca gctgcctcac

tgaaaaggag gacagcccca gtgaaagcac aggaaatgga ccccccccacc tggcccaccc

aaacctggac acgtttaccc cggaggagct gctgcagcag atgaaagagc tcctgaccga

gaaccaccag ctgaaaggtg agcagggctg gcccctgtgt gccccattca tcctgggcct

Sequence of OPTN gene, GeneBank Accession No. AF423071

```
   1 atcccggtcg ggagttctct ccaggcggca cgatgccgag gaaacagtga ccctgagcga
  61 agccaagccg ggcggcaggt gtggctttga tagctggtgg tgccacttcc tggccttgga
 121 tgagccgtac gcctctgtaa acccaacttc ctcacctttg aaacagctgc ctggttcagc
 181 attaatgaag attagtcagt gacaggcctg gtgtgctgag tccgcacata gaagaatcaa
 241 aaatgtccaa aatgtaactg gagagaaagt gggcaacttt tggagtgact tttccacagg
 301 aacttctgca atgtcccatc aacctctcag ctgcctcact gaaaaggagg acagccccag
 361 tgaaagcaca ggaaatggac ccccccacct ggcccaccca aacctggaca cgtttacccc
 421 ggaggagctg ctgcagcaga tgaaagagct cctgaccgag aaccaccagc tgaaagaagc
 481 catgaagcta aataatcaag ccatgaaagg gagatttgag gagctttcgg cctggacaga
 541 gaaacagaag gaagaacgcc agtttttga gatacagagc aaagaagcaa aagagcgtct
 601 aatggccttg agtcatgaga atgagaaatt gaaggaagag cttggaaaac taaaagggaa
 661 atcagaaagg tcatctgagg accccactga tgactccagg cttcccaggg ccgaagcgga
 721 gcaggaaaag gaccagctca ggacccaggt ggtgaggcta caagcagaga aggcagacct
 781 gttgggcatc gtgtctgaac tgcagctcaa gctgaactcc agcggctcct cagaagattc
 841 ctttgttgaa attaggatgg ctgaaggaga agcagaaggg tcagtaaaag aaatcaagca
 901 tagtcctggg cccacgagaa cagtctccac tggcacggca ttgtctaaat ataggagcag
 961 atctgcagat ggggccaaga attacttcga acatgaggag ttaactgtga gccagctcct
1021 gctgtgccta agggaaggga atcagaaggt ggagagactt gaagttgcac tcaaggaggc
1081 caaagaaaga gtttcagatt ttgaaaagaa aacaagtaat cgttctgaga ttgaaaccca
```

1141 gacagagggg agcacagaga aagagaatga tgaagagaaa ggcccggaga ctgttggaag

1201 cgaagtggaa gcactgaacc tccaggtgac atctctgttt aaggagcttc aagaggctca

1261 tacaaaactc agcgaagctg agctaatgaa gaagagactt caagaaaagt gtcaggccct

1321 tgaaaggaaa aattctgcaa ttccatcaga gttgaatgaa aagcaagagc ttgtttatac

1381 taacaaaaag ttagagctac aagtggaaag catgctatca gaaatcaaaa tggaacaggc

1441 taaaacagag gatgaaaagt ccaaattaac tgtgctacag atgacacaca acaagcttct

1501 tcaagaacat aataatgcat tgaaaacaat tgaggaacta acaagaaaag agtcagaaaa

1561 agtggacagg gcagtgctga aggaactgag tgaaaaactg gaactggcag agaaggctct

1621 ggcttccaaa cagctgcaaa tggatgaaat gaagcaaacc attgccaagc aggaagagga

1681 cctggaaacc atgaccatcc tcagggctca gatggaagtt tactgttctg attttcatgc

1741 tgaaagagca gcgagagaga aaattcatga ggaaaaggag caactggcat tgcagctggc

1801 agttctgctg aaagagaatg atgctttcga agacggaggc aggcagtcct tgatggagat

1861 gcagagtcgt catggggcga gaacaagtga ctctgaccag caggcttacc ttgttcaaag

1921 aggagctgag gacagggact ggcggcaaca gcggaatatt ccgattcatt cctgccccaa

1981 gtgtggagag gttctgcctg acatagacac gttacagatt cacgtgaatgg attgcatcat

2041 ttaagtgttg atgtatcacc tccccaaaac tgttggt

Partial nucleotide sequence for TNF-α gene comprising the targeted polymorphic position is as follows:

TNF-α -863C>A; -857C>T (underlined)

3121 ccacatgtag cggctctgag gaatgggtta caggagacct ctggggaagat gtgaccacag

3181 caatgggtag gagaatgtcc agggctatga aagtcgagta tggggacccc ccttaacga

-863C>A -857C>T

3241 agacagggcc atgtagaggg ccccagggag tgaaagagcc tccaggaacct ccaggtatgg

3301 aatacagggg acgtttaaga agatatggcc acacactggg gccctgaagaa gtgagagctt

Example 7. Effect of Oral Angiotensin II Receptor Blocker on IOP in Normal Subjects and Its Association with SNPs in AT1R and AT2R Genes

Example 7-1.

Methods

Relationship between polymorphism at nucleotide number 3123 (C or A) of the angiotensin II receptor 2 gene (AT2R) on chromosome-X and the effect of candesartan cilexetil, an angiotensin II receptor blocker was examined. This study was performed on 20 healthy volunteers (13 men and 8 women) without systemic and eye diseases. Among them, 9 men had C, 4 men had A, 4 women had CC and 4 women had CA genotype at the polymorphic point. The each subject was given candesartan cilexetil orally and the IOP was recorded from 1 to 24 hours after the administration.

RESULTS

Change in Intraocular pressure 1-24 hours after the drug administration is shown in Table 19.

Table 19.

time 0	Lowering IOP mmHg							AT2R 3123C>A				
Base Line	1 Hr	2 Hr	3 Hr	4 Hr	5 Hr	6 Hr	24 Hr	M	M	F	F	
0	-2	-1	-3	-2	-1	-1	-1		A			
0	-2	-2	0	0	-1	1	1		A			I
0	1	1	0	0	-2	-2	0		A			
0	0	0	-2	1	0	0	-1	C				
0	-1	-3	-5	-2	-3	-3	-3	C				
0	0	-3	-2	-4	-3	0	0				CA	
0	-1	-1	-4	-3	-4	-3	1	C				II
0	-2	-4	-4	-4	-4	-5	-2	C				
0	-2	-3	-3	-2	-2	1	2			CC		
0	-2	-3	-2	-5	-3	-3	0	C				
0	-4	-6	-6	-6	-6	-4	-5				CA	
0	-4	-5	-6	-5	-5	-5	-7	C				
0	-4	-6	-6	-8	-5	-5	-4				CA	
0	-2	-3	-6	-5	-6	-3	-3	C				
0	-2	-4	-4	-6	-3	-4	-5				CA	
0	-4	-8	-6	-7	-6	-6	-2			CC		III
0	-4	-4	-5	-3	-5	-4	-3	C				
0	-1	-4	-6	-3	-6	-4	0			CC		
0	-2	-4	-7	-5	-7	-6	-3			CC		
0	-2	-7	-6	-4	-6	-6	-1	C				
0	-6	-8	-8	-12	-12	-12	-12		A			

	IOP Lowering Effect	genotype
Group I	-	3 of 4 had A
Group II	+	5 of 6 had C or CC
Group III	++	7 of 11 had C or CC

In male, oral administration of candesartan cilexetil hardly lowered the IOP of 75% of those with A genotype at nucleotide 3123 of AT2R gene, whereas the IOP of 100% of those with C genotype was effectively lowered. In female, oral administration of candesartan cilexetil was effectively lower the IOP of 100% of those with CC genotype.

This result suggest that nucleotide 3123 of AT2(AGTR2) gene polymorphism associate with the effect of candesartan cilexetil.

Example 7-2.

Methods

This study was performed on 20 healthy volunteers (13 men and 7 woman, age 23 to 28 years) without systemic and

eye diseases. In the morning (10:00 hr), each subject was given either 12 mg oral candesartan cilexetil (Blopress®, Takeda, Japan) or the placebo in a randomized crossover double-blind fashion.

The baseline heart rate, systolic/diastolic arterial pressures (SBP/DBP), and IOP were recorded. The subjects then received oral candesartan cilexetil or placebo, and measurements were repeated hourly for 6 hr and after 24 hr. One month later, each subject received the alternative treatment. Only the right eye was measured and analyzed.

The ocular perfusion pressure (OPP) is defined as the difference between the pressure in the arteries entering the tissue and the veins leaving it. The OPP can be approximated by the following formula using the mean blood pressure (BPm) and the IOP.

OPP = 2/3 x BPm - IOP, where BPm = DBP + 1/3 x (SBP - DBP).

A search for polymorphisms in ATR1 and ATR2 was performed in the 20 subjects and correlated with the changes in the IOP. This research followed the tenets of the Declaration of Helsinki. Written informed consent was obtained after the nature and possible consequences of the study were explained. Where applicable, the research was approved by the institutional human experimentation committee for analysis of DNA.

Statistical Analysis

Statistical analysis of the results following ARB was performed with StatView (SAS Institute, USA) using repeated measure ANOVA test. ANOVA test with Bonferroni correction was used for statistical analysis of each IOP values: a *P* value <0.0004 was considered to be statistically significant.

RESULTS

The changes in the IOP after oral candesartan cilexetil or placebo are shown in **Figure 5A**. The IOP in the subjects who received the placebo was not altered significantly. On the other hand, as early as 1 hr after oral candesartan cilexetil, the IOP had fallen significantly and remained low for 5 hr (*P* <0.0001) compared with placebo. Candesartan cilexetil did not significantly affect perfusion pressures (**Fig. 5B**). No significant change in SBP, DBP, and heart rate was detected after a single oral dose of candesartan cilexetil or placebo (data not shown).

The changes in the IOP after oral candesartan cilexetil in each of the 20 subjects are shown in **Figure 5C**. There was no significant association between the effects of candesartan cilexetil and the three SNPs in the ATR1 gene in the 20 control subjects (**Table 19-2**). For the ATR2 genotype, however, 4 men with the A genotype showed a

reduction of the IOP by 2.3 ± 0.5 mmHg, which was the same value as that of subjects who received placebo, and a significantly less decrease in the IOP than in the 9 men with the C genotype (5.0 ± 1.1 mmHg, P = 0.014). No woman had the AA genotype in this study.

Table 19-2. Effects of angiotensin II receptor blocker on intraocular pressure in association with genotypes of the angiotensin II receptor genes

Polymorphisms	Genotype	Number (eyes)	Maximum reduction of IOP (mmHg)	P*
AGTR1 -713T>G	TT	18	4.9 ± 1.8	
	TG	2	5.0 ± 4.2	P=0.898
	GG	0	0	
AGTR1 -521C>T	CC	18	4.9 ± 1.8	
	CT	1	2	P† =0.117
	TT	1	8	
AGTR1 1166A>C	AA	18	5.1 ± 2.0	
	AC	2	5.2 ± 1.6	P=0.405
	CC	0	0	
AGTR2 3123C>A	C (male)	9	5.0 ± 1.1	P=0.014 ‡
	A (male)	4	2.3 ± 0.5	
	CC (female)	3	7.0 ± 1.0	
	CA (female)	4	6.0 ± 1.6	P=0.354
	AA (female)	0	0	

* P value for Mann-Whitney U test
† P value for Kruskal-Wallis test
‡ P <0.05

Example 8. Associations between glaucoma and gene polymorphisms of endothelin-1 and endothelin type A receptor

Purpose: Endothelin 1 (ET-1), a potent vasoconstrictor, may affect regulation of intraocular pressure and ocular vessel

tone. Thus, ET-1 and its receptors may contribute to development of glaucoma. We investigated whether gene polymorphisms of ET-1 (*EDN1*) and its receptors ET_A (*EDNRA*) and ET_B (*EDNRB*) were associated with glaucoma phenotypes and clinical features.

Methods

Study population:

A total of 650 Japanese subjects (224 normal controls, 176 POAG patients, and 250 NTG patients), recruited from seven Japanese medical institutions, were examined in this study. All subjects were unrelated. Mean age (± standard deviation) at diagnosis of OAG was 57.2±12.8 years. OAG subjects were divided into POAG patients and NTG patients, aged 58.8±12.2 and 56.1±13.2 years at diagnosis, respectively (Table 1). Mean age at the time of examination was 70.0±11.2 years in controls. We purposely selected older control subjects to reduce the likelihood that a subset of controls would later develop glaucoma.

Ophthalmic examinations included slit-lamp biomicroscopy, optic disc examination, IOP measurement by Goldmann applanation tonometry, and gonioscopy. Visual fields were assessed with Humphrey automated perimetry (program 30-2) or Goldmann perimetry. Severity of visual field defects was scored from 1 to 5. Data obtained by two

types of perimetry were combined using a five-point scale: 1, no alterations; 2, early defects; 3, moderate defects; 4, severe defects; and 5, light perception only or no light perception. This severity scale followed Kozaki's classification, which has been used most widely in Japan so far, based on Goldmann perimetry, or by the classification established for the Humphrey Field Analyzer.

POAG was diagnosed on fulfillment of all of the following criteria: maximum IOP was above 21 mm Hg; open angles on gonioscopy; typical glaucomatous disc cupping associated with visual field changes; and absence of other ocular, rhinologic, neurological, or systemic disorders potentially causing optic nerve damage. We excluded patients with elevated IOP secondary to defined causes (e.g., trauma, uveitis, steroid administrtion, or exfoliative, pigmentary, or neovascular glaucoma). POAG patients with *MYOC* mutations and JOAG patients were also excluded. NTG was diagnosed by the same criteria as POAG except that IOP did not exceed 21 mm Hg at all times during the follow-up period. Normal control subjects had IOP less than 20 mm Hg, no glaucomatous disc changes, and no family history of glaucoma.

DNA extraction and genotyping of the polymorphisms

Genomic DNA was isolated from peripheral blood lymphocytes by standard methods. Nine single nucleotide

polymorphisms (SNPs) were detected among all participants: four for *EDN1* (T-1370G, +138/ex1 del/ins, G8002A, K198N); four for *EDNRA* (G-231A, H323H, C+70G, C+1222T); and one for *EDNRB* (L277L). These polymorphisms are listed at http://genecanvas.idf.inserm.fr/. We genotyped these SNPs using the Invader® assay (Third Wave Technologies, Inc, Madison, WI), which was recently developed for high-throughput genotyping of SNPs (Lyamichev V et. al., Nat Biotechnol 1999;17:292-296, the contents of the cited reference are herein incorporated by reference).

The oligonucleotide sequences of primary probes and Invader® probes used in this study are listed in **Table 20.**

Table 20. Sequences of primary probes and Invader oligonucleotides used in assays

Polymorphism	Location	Nucleotide change	Target	Probe	Sequence (The lower case letters indicate the flap sequences)
EDN1/T-137G	5'-flanking region	T/G	Anti-sense	T probe	Flap sequence-TTGGTGGAGAACAAACAA
				G probe	Flap sequence-GTGGTGGAGAACAAACA
				Invader	GGTGTTACTGGGCCACTGTGAGCGCTC
EDN1/+138 del/ins	Exon 1	del/ins	Sense	A del probe	Flap sequence-TAACGGGGAGAAAAGG
				A ins probe	Flap sequence-TTAACGGGGAGAAAAGG
				Invader	GCGATCCTTCAGCCCAAGTGCCCTTC
EDN1/G8002A	Intron 4	G/A	Anti-sense	G probe	Flap sequence-GAAAATCATTTTGGGGAGC
				A probe	Flap sequence-AAAAATCATTTTGGGGAGC
				Invader	TGCCTCTCTGAGTGAAATGTATTTACCACTTTCCCTGAGAAATCT
EDN1/K198N	Exon 5	G/T	Sense	G probe	Flap sequence-CTTGCCTTTCAGCTTGG
				T probe	Flap sequence-ATTGCCTTTCAGCTTGG
				Invader	GTTGTGCGGTCACATAACGCCTCTCTGGAGGGT
EDNRA/G-231A	Exon 1	G/A	Sense	G probe	Flap sequence-CTCCTGCGCACTGC
				A probe	Flap sequence-TTCCTGCGCACTGC
				Invader	CTGCACAGCTTCCCCGCCTCAGAAAACA
EDNRA/H323H	Exon 6	T/C	Anti-sense	T probe	Flap sequence-TTTAAGCGCTATATTGAAGAAAA
				C probe	Flap sequence-CTTAAGCGCTATATTGAAGAAAA
				Invader	CTTGGTTGTAATTTTTGCTCTTGCTGGTTCCCTCTTCAA
EDNRA/C+70G	Exon 8	C/G	Sense	C probe	Flap sequence-GTCACAGTTGCCTTGT
				G probe	Flap sequence-CTCACAGTTGCCTTGT
				Invader	GGAAGAAGGATCAGAGAAGAGATTCCCGGAT
EDNRA/C+1222T	Exon 8	C/T	Anti-sense	C probe	Flap sequence-CTTGCGGTTTTCAGTATGA
				T probe	Flap sequence-TTTGGGGTTTTCAGTATGA
				Invader	CCCACAAATGCCACCACAGAACTTAACGATTCTTCACTTA
EDNRB/L277L	Exon 4	A/G	Anti-sense	A probe	Flap sequence-ATTCAGTTTCTATTTCTGTTG
				G probe	Flap sequence-GTTCAGTTTCTATTTCTGTTG
				Invader	CTCATCCCTATAGTTTTACAAGACAGCAAAAAGATTGGTGCTT

Nine polymorphisms were detected among all participants. These polymorphisms are listed at http://genecanvas.ldf.free.nl.fr/. Genotyping of the polymorphisms were performed by the Invader® assay using the probes listed above.

Statistical analysis

Comparisons of genotype distributions in normal

controls with those in OAG patients, POAG patients, and NTG patients were performed by χ^2 analysis. Associations of clinical characteristics (age at diagnosis, untreated maximum of IOP, and visual field score at diagnosis) with genotypes were assessed by the Mann-Whitney *U* test. Statistical analyses were carried out with SPSS for Windows (version 12.0; SPSS Inc, Chicago, IL). A value of $p<0.05$ was considered to be significant.

Results

Table 21 shows genotype and allele frequencies obtained in this study. Distributions were consistent with Hardy-Weinberg equilibrium. For the *EDN1*/+138/ex1 del/ins polymorphism, frequencies of the del/del and del/ins + ins/ins genotypes respectively were 74.2% and 25.8% in OAG patients overall ($p=0.016$), 74.4% and 25.6% in POAG patients ($p=0.047$), and 74.0% and 26.0% in NTG patients ($p=0.037$), compared with 65.2% and 34.8% in control subjects. For the *EDN1*/K198N polymorphism, 53.2% of OAG patients were found to have the KK genotype, which was significantly higher than the 43.8% prevalence in control subjects ($p=0.022$). When OAG patients were divided into those with POAG and those with NTG, frequency of the KK genotype in NTG patients was much higher than in controls ($p=0.008$), while genotype and allele frequency distributions in POAG patients did not differ statistically

from those in controls. A gender difference was noted; specifically, the KK genotype was significantly more prevalent in female NTG patients (p=0.010 vs. female controls) than in male NTG patients (p=0.251 vs. male controls; **Table 22**). Polymorphism of *EDN1*/G8002A in the intron 4 region was highly coincident with *EDN1*/K198N, except in one sample (data not shown).

Frequencies of *EDNRA*/C+1222T genotypes (CC vs. CT+TT) differed slightly between OAG patients and controls (p=0.036). Distribution of genotypes for other polymorphisms showed no significant differences between any patient group and controls.

Characteristics of patients are examined in dominant model and recessive model of each polymorphism, and data with significant differences are shown in **Table 23**. In OAG patients overall and in POAG patients, no characteristic showed a significant difference between genotype groups. In NTG patients, however, the AA group of *EDNRA*/G-231A had poorer visual field scores at diagnosis than the GG+GA group (3.0±0.8 vs. 2.7±0.6, p=0.043). We also found significantly poorer visual field scores at diagnosis in the GG group for *EDNRA*/C+70G than the CC+CG group among NTG patients (3.0±0.7 vs. 2.7±0.7, p=0.014). Untreated maximum of IOP in the TT group for *EDNRA*/H323H was statistically higher than in the CC+CT group in NTG patients (17.2±2.2 vs.

16.6±2.3, p=0.040). Other polymorphisms in NTG patients showed no significant differences in characteristics between genotype groups.

Table 21. Genotype and allele frequencies of EDN1, EDNRA, and EDNRB polymorphisms in control subjects and glaucoma patients

Polymorphism		Genotype frequency		p value	Allele frequency		p value
		TT	TG+GG		T	G	
EDN1/T−1370G	Control (n=224)	133 (59.4)	91 (40.6)		350 (78.1)	98 (21.9)	
	OAG (n=426)	273 (64.1)	153 (35.9)	0.239	675 (79.2)	177 (20.8)	0.644
	POAG (n=176)	108 (61.4)	68 (38.6)	0.687	275 (78.1)	77 (21.9)	1.000
	NTG (n=250)	165 (66.0)	85 (34.0)	0.136	400 (80.0)	100 (20.0)	0.478
		del del	del ins + ins ins		del	ins	
EDN1/+138/ex1 del/ins	Control (n=224)	146 (65.2)	78 (34.8)		364 (81.3)	84 (18.8)	
	OAG (n=426)	316 (74.2)	110 (25.8)	0.016*	734 (86.2)	118 (13.8)	0.020*
	POAG (n=176)	131 (74.4)	45 (25.6)	0.047*	303 (86.1)	49 (13.9)	0.069
	NTG (n=250)	185 (74.0)	65 (26.0)	0.037*	431 (86.2)	69 (13.8)	0.039*
		KK	KN+NN		K	N	
EDN1/K198N	Control (n=224)	98 (43.8)	126 (56.3)		295 (65.8)	153 (34.2)	
	OAG (n=425)	226 (53.2)	199 (46.8)	0.022*	609 (71.6)	241 (28.4)	0.031*
	POAG (n=175)	86 (49.1)	89 (50.9)	0.284	245 (70.0)	105 (30.0)	0.213
	NTG (n=250)	140 (56.0)	110 (44.0)	0.008*	364 (72.8)	136 (27.2)	0.020*
		GG	GA+AA		G	A	
EDNRA/G−231A	Control (n=224)	62 (27.7)	162 (72.3)		244 (54.5)	204 (45.5)	
	OAG (n=425)	118 (27.8)	307 (72.2)	0.981	455 (53.5)	395 (46.5)	0.748
	POAG (n=176)	52 (29.5)	124 (70.5)	0.681	195 (55.4)	157 (44.6)	0.792
	NTG (n=249)	66 (26.5)	183 (73.5)	0.774	260 (52.2)	238 (47.8)	0.488
		TT	TC+CC		T	C	
EDNRA/H323H	Control (n=224)	122 (54.5)	102 (45.5)		327 (73.0)	121 (27.0)	
	OAG (n=426)	228 (53.5)	198 (46.5)	0.819	626 (73.5)	226 (26.5)	0.852
	POAG (n=176)	95 (54.0)	81 (46.0)	0.923	259 (73.6)	93 (26.4)	0.852
	NTG (n=250)	133 (53.2)	117 (46.8)	0.783	367 (73.4)	133 (26.6)	0.887
		CC	CG+GG		C	G	
EDNRA/C+70G	Control (n=224)	61 (27.2)	163 (72.8)		229 (51.1)	219 (48.9)	
	OAG (n=426)	128 (30.0)	298 (70.0)	0.453	462 (54.2)	390 (45.8)	0.286
	POAG (n=176)	57 (32.4)	119 (67.6)	0.262	196 (55.7)	156 (44.3)	0.199
	NTG (n=250)	71 (28.4)	179 (71.6)	0.777	266 (53.2)	234 (46.8)	0.521
		CC	CT+TT		C	T	
EDNRA/C+1222T	Control (n=224)	137 (61.2)	87 (38.8)		347 (77.5)	101 (22.5)	
	OAG (n=426)	224 (52.6)	202 (47.4)	0.036*	620 (72.8)	232 (27.2)	0.066
	POAG (n=176)	92 (52.3)	84 (47.4)	0.074	254 (72.2)	98 (27.8)	0.085
	NTG (n=250)	132 (52.8)	118 (47.2)	0.067	366 (73.2)	134 (26.8)	0.130
		AA	AG+GG		A	G	
EDNRB/L277L	Control (n=224)	77 (34.4)	147 (65.6)		254 (56.7)	194 (43.3)	
	OAG (n=425)	118 (27.8)	307 (72.2)	0.081	443 (52.1)	407 (47.9)	0.116
	POAG (n=176)	48 (27.3)	128 (72.7)	0.128	184 (52.3)	168 (47.7)	0.212
	NTG (n=249)	70 (28.1)	179 (71.9)	0.142	259 (52.0)	239 (48.0)	0.148

Data are n (%).

* $P < 0.05$ (χ^2 test).

Genotype distributions showed significant differences for *EDN1*/+138/ex1 del/ins (p=0.016) and *EDN1*/K198N (p=0.022) polymorphisms, and a slight difference for *EDNRA*/C+1222T polymorphism (p=0.036) between OAG patients and controls. After dividing the OAG group into POAG and NTG, frequency of the KK genotype for the EDN1/K198N polymorphism in NTG patients was much higher than in controls (p=0.008).

Table 22. Genotype frequency of EDN1/K198N polymorphism in male and female subjects

Polymorphism	Male	Genotype frequency		p value	Female	Genotype frequency		p value
		KK	KN+NN			KK	KN+NN	
EDN1/K198N	Control (n=100)	46 (46.0)	54 (54.0)		Control (n=124)	52 (41.9)	72 (58.1)	
	OAG (n=218)	112 (51.4)	106 (48.6)	0.373	OAG (n=207)	114 (55.1)	93 (44.9)	0.021*
	POAG (n=99)	48 (48.5)	51 (51.5)	0.726	POAG (n=76)	38 (50.0)	38 (50.0)	0.266
	NTG (n=119)	64 (53.8)	55 (46.2)	0.251	NTG (n=131)	76 (58.0)	55 (42.0)	0.010*

Data are n (%).

* $P < 0.05$ (χ^2 test).

In the *EDN1*/K198N polymorphism, genotype distributions diversed according to gender. The KK genotype for this polymorphism was significantly more prevalent in female NTG patients (p=0.010 vs. female controls) than in male NTG patients (p=0.251 vs. male controls).

Table 23. Characteristics of glaucoma patients according to genotype

Polymorphism	Type of glaucoma	Characteristic	Genotype		p value
			GG+GA	AA	
EDNRA/G-231A	NTG	Age at diagnosis (years)	56.9±13.1 (n=192)	53.6±13.5 (n=55)	0.102
		Untreated maximum IOP (mm Hg)	17.1 ±2.3 (n=188)	16.4±2.2 (n=52)	0.052
		Visual field score at diagnosis	2.7±0.6 (n=194)	3.0±0.8 (n=55)	0.043*
			TT	TC+CC	
EDNRA/H323H	NTG	Age at diagnosis (years)	55.7±13.5 (n=131)	56.6±12.9 (n=117)	0.508
		Untreated maximum IOP (mm Hg)	17.2±2.2 (n=129)	16.6±2.3 (n=112)	0.040*
		Visual field score at diagnosis	2.8±0.7 (n=133)	2.7±0.7 (n=117)	0.307
			CC+CG	GG	
EDNRA/C+703	NTG	Age at diagnosis (years)	55.7±13.3 (n=194)	57.8±12.7 (n=54)	0.373
		Untreated maximum IOP (mm Hg)	17.0±2.2 (n=188)	16.5±2.3 (n=53)	0.141
		Visual field score at diagnosis	2.7±0.7 (n=195)	3.0±0.7 (n=55)	0.014*

Data are means ±SD.
* $P < 0.05$ (Mann-Whitney U test).

The AA genotype of *EDNRA*/G-231A and the GG genotype of *EDNRA*/C+70G were associated with worse visual field defects in NTG patients (p=0.043 and 0.014, respectively). The *EDNRA*/H323H polymorphism influenced untreated maximum IOP among NTG patients (p=0.040).

In male subjects, the following correlations were confirmed:

1) The A138insertion/deletion(A138I/D) polymorphism in exon 1 of the Endothelin-1 gene is associated with both of POAG and NTG (Table 24).

2) The -231A>G polymorphism of promoter region of the Endothelin receptor A gene is associated with NTG, especially with patients with intraocular pressure at less than 15mmHg (Table 25).

3) The CAC to CAT substitution at codon No. 233 in exon 6 of the Endothelin receptor A gene (His323His) is associated with NTG, especially with patients with intraocular pressure at less than 15mmHg (Table 26).

4) The CTG to CTA substitution at codon No. 277 in exon 4 of the Endothelin receptor B gene is associated with both of POAG and NTG (Table 27).

In female patients, following correlations were confirmed:

1) The AAG to AAT substitution at codon No. 198 of the endothelin-1 gene (Lys198Asn) is associated with NTG (Table 28).

2) The -1370T>G polymorphism of the Endothelin-1 gene promoter region is associated with NTG(Table 29).

3) The +70C>G(70 bases from the stop codon) polymorphism in 3' non-coding region of the Endothelin receptor A is associated with POAG (Table 30).

4) The +1222C>T(1222 bases from the stop codon) polymorphism in 3' non-coding region of the Endothelin receptor A is associated NTG(wherein the intraocular pressure is 16mmHg-21mmHg) (Table 31).

Table 24. Endothelin A138I/D (Male)

	N	Genotype Frequency			p	Genotype Frequency		p	Genotype Frequency		χ^2 test p
		I/I	I/D	D/D		I/I	I/D+D/D		I/I+I/D	D/D	
Control	100	4	34	62		4	96		38	62	
POAG	100	3	21	76		3	97		24	76	0.032
NTG	119	1	28	90		1	118		29	90	0.029

Table 25. Endothelin Receptor A -231A>G (Male)

	N	Genotype Frequency			p	Genotype Frequency		p	Genotype Frequency		χ^2 test p
		AA	AG	GG		AA	AG+GG		AA+AG	GG	
Control	100	22	45	33		22	78		67	33	
POAG	100	24	51	25		24	76		75	25	
NTG	119	30	60	29		30	89		90	29	
H-NTG	89	17	45	27		17	72		62	27	
L-NTG	25	11	12	2	0.017	11	14	0.026	23	2	0.025

H-NTG: NTG patients with intraocular pressure at 16 mmHg-21mmHg.

L-NTG: NTG patients with maximal intraocular pressure at 15mmHg or less.

Table 26. Endothelin Receptor A H323H C>T His323His (Male)

	N	Genotype Frequency			p	Genotype Frequency		p	Genotype Frequency		χ^2 test p
		CC	CT	TT		CC	CT+TT		CC+CT	TT	
Control	100	9	40	51		9	91		49	51	
POAG	100	7	38	55		7	93		45	55	
NTG	119	11	50	58		11	108		61	58	
H-NTG	89	7	32	50		7	82		39	50	
L-NTG	25	4	14	7		4	21		18	7	0.039

H-NTG: NTG patients with intraocular pressure at 16 mmHg-21mmHg.

L-NTG: MTG patients with maximal intraocular pressure at

15mmHg or less.

Table 27. Endothelin Receptor B L277L G>A Leu277Leu (Male)

	n	Genotype Frequency			p	Genotype Frequency		p	Genotype Frequency		χ^2 test p
		GG	GA	AA		GG	GA+AA		GG+GA	AA	
Control	100	18	41	41		18	82		59	41	
POAG	100	26	48	26		26	74		74	26	0.025
NTG	119	26	61	32		26	93		87	32	0.027

Table 28. Endothelin Lys198Asn G>T or K198N (Female)

	N	Genotype Frequency			p	Genotype Frequency		p	Genotype Frequency		χ^2 test p
		KK	KN	NN		KK	KN+NN		KK+KN	NN	
Control	124	52	59	13		52	72		111	13	
POAG	76	38	33	5		38	38		71	5	
NTG	131	76	38	17	0.009	76	55	0.010	114	17	

Table 29. Endothelin -1370T>G (Female)

	N	Genotype Frequency			p	Genotype Frequency		p	Genotype Frequency		χ^2 test p
		TT	TG	GG		TT	TG+GG		TT+TG	GG	
Control	124	66	56	2		66	58		122	2	
POAG	76	49	24	3		49	27		73	3	
NTG	131	84	39	8	0.013	84	47		123	8	

Table 30. Endothelin Receptor A +70C>G (Female)

	N	Genotype Frequency			p	Genotype Frequency		p	Genotype Frequency		χ^2 test p
		CC	CG	GG		CC	CG+GG		CC+CG	GG	
Control	124	29	59	36		29	95		88	36	
POAG	76	28	32	16		28	48	0.041	60	16	
NTG	131	35	66	30		35	96		101	30	

Table 31. Endothelin Receptor A +1222C>T (Female)

	N	Genotype Frequency			p	Genotype Frequency		p	Genotype Frequency		χ^2 test p
		CC	CT	TT		CC	CT+TT		CC+CT	TT	
Control	124	74	42	8		74	50		116	8	
POAG	76	40	30	6		40	36		70	6	
NTG	131	66	54	11		66	65		120	11	
H-NTG	92	42	42	8		42	50	0.041	84	8	
L-NTG	35	21	11	3		21	14		32	3	

H-NTG: NTG patients with intraocular pressure at 16 mmHg-21mmHg.

L-NTG: MTG patients with maximal intraocular pressure at 15mmHg or less.

Partial nucleotide sequences of endothelin-1(EDN1) and endothelin receptor A (EDNRA) and endothelin receptor B (EDNRB) comprising the targeted polymorphisms are shown below

EDN1 -1370 (underlined) T>G

2101 ttgaattcca ccctccatcc ccagaaaaac tggagtaaaa caaaaagagg agatggacaa

2161 agtgtgtatt tgatggcatc ccctgggaag agactctaaa tttatcccat aggtcttact

2221 gggccactgt gagcgctttg gtggagaaca aacaaaaatt ctgggtgctc agttgtctaa

2281 cctgaaaaat gggactagcg gaaaaagcca atgtgttcca tgcacctttt gctttcttta

2341 ttaaggcatg atgtcacctg tacagtaact gccctgtgtg tacttcaggg

END1 +138 (underlined) ins/del(each one of the a at 3743-3745)

3661 ccagctctcc accgccgcgt gcgcctgcag acgctccgct cgctgccttc tctcctggca

3721 ggcgctgcct tttctccccg ttaaagggca cttgggctga aggatcgctt tgagatctga

3781 ggaacccgca gcgctttgag ggacctgaag ctgtttttct tcgttttcct ttgggttcag

3841 tttgaacggg aggtttttga tccctttttt tcagaatgga ttatttgctc atgattttct

(atg is the initiation codon)

EDNRA +70(underlined) C>G

63601 atccagtgga agaaccacga tcaaaacaac cacaacacag accggagcag ccataaggac

63661 agcatgaact gaccaccctt agaagcactc ctcggtactc ccataatcct ctcggagaaa

63721 aaaatcacaa ggcaactgtg actccgggaa tctcttctct gatccttctt ccttaattca

63781 ctcccacacc caagaagaaa tgctttccaa aaccgcaagg gtagactggt ttatccaccc

63841 acaacatcta cgaatcgtac ttctttaatt gatctaattt acatattctg cgtgttgtat

(tga is the translation termination codon)

EDNRA +1222(underlined) C>T

64741 ttaatttttc ttaaaatgtt aactggcagt aagtcttttt tgatcattcc cttttccata

64801 taggaaacat aattttgaag tggccagatg agtttatcat gtcagtgaaa aataattacc

64861 cacaaatgcc accagaactt aacgattctt cacttcttgg ggttttcagt atgaacctaa

64921 ctccccaccc caacatctcc ctcccacatt gtcaccattt caaagggccc acagtgactt

64981 ttgctgggca ttttcccaga tgtttacaga ctgtgagtac agcagaaaat cttttactag

EDNRA codon No. 323 (underlined) (T>C) His323His

60721 gaggtagagg cagtgtaagc caggctgttc tcctggctct tctttgaatt attctttctc

60781 tggtgtctgc tacttcttgg tactgtagtt cttgcatcta gtataaaaac actaaatttg

60841 ttgtcctatt ttttttctcac tttcctttag cgtcgagaag tggcaaaaac agttttctgc

60901 ttggttgtaa ttttttgctct ttgctggttc cctcttcatt taagccgtat attgaagaaa

60961 actgtgtata acgagatgga caagaaccga tgtgaattac ttaggtatga tcctgtgtac

61021 tcgctagaaa attggagttt ctcagatttt catatttata atacttttac aaaaccagct

EDNRA -231(underlined) A>G

2041 ggaggagacg gggaggacag actggaggcg tgttcctccg gagttttctt tttcgtgcga

2101 gccctcgcgc gcgcgtacag tcatcccgct ggtctgacga ttgtggagag gcggtggaga

2161 ggcttcatcc atcccacccg gtcgtcgccg gggattgggg tcccagcgag acctccccgg

2221 gagaagcagt gcccaggagg ttttctgaag ccggggaagc tgtgcagccg aagccgccgc

2281 cgcgccggag cccgggacac cggccaccct ccgcgccacc caccctcgcc ggctccggct

2341 tcctctggcc caggcgccgc gcggacccgg cagctgtctg cgcacgccga gctccacggt

EDNRB codon No. 277(underlined) Leu277Leu (CTG to CTA)

75361 taatcattcc ctgatgaatt tttttaagtt taacatttgt tatataagat tttcttacag

75421 aggagtatta atcgtaaaaa ttctctcatc cctatagttt tacaagacag caaaagattg

75481 gtggctgttc agtttctatt tctgcttgcc attggccatc actgcatttt tttatacact

75541 aatgacctgt gaaatgttga gaaagaaaag tggcatgcag attgctttaa atgatcacct

75601 aaagcaggta agaaaataca aatatttgat aactcgtggt tgaatttata attatgaata

Example 9. Association between Gene Polymorphism of β1 adrenergic receptor (ADRB1) and Glaucoma

Methods

Association between gene polymorphism of ADRB1 and glaucoma was examined among POAG, NTG patients and normal (control) subjects using PCR-RFLP techniques (Table 32-1).

Table 32-1. Primer sequences

Gene		Primer sequences	Restriction Enzyme
ADRB1 Gly389Arg	F	CCG CCT CTT CGT CTT CTT CAA CTG	BsmF1
	R	GAT AGC AGG TGA ACT CGA AGC CCA	

Results

As shown in Table 32-2, the polymorphism of Gly389Arg in ADRB1 is associated with NTG (Table 32-2).

Table 32-2. β1-Adrenalin Receptor Gly389Arg

	N	Genotype Frequency			p	Genotype Frequency		p	Genotype Frequency		χ^2 test p
		CC	CG	GG		CC	CG+GG		CC+CG	GG	
Control	240	147	78	15		147	93		225	15	
POAG	191	127	58	6		127	64		185	6	
NTG	284	197	80	7	0.038	197	87		277	7	0.031

Partial nucleotide sequence of β1-Adrenalin Receptor comprising the targeted polymorphism.

B1AR codon 389(underlined GGA(Gly) to CGA(Arg) Gly389Arg

1021 ttcctggcca acgtggtgaa ggccttccac cgcgagctgg tgcccgaccg cctcttcgtc

1081 ttcttcaact ggctgggcta cgccaactcg gccttcaacc ccatcatcta ctgccgcagc

1141 cccgacttcc gcaaggcctt ccagggactg ctctgctgcg cgcgcagggc tgcccgccgg

1201 cgccacgcga cccacggaga ccggccgcgc gcctcgggct gtctggcccg gcccggaccc

1261 ccgccatcgc ccggggccgc ctcggacgac gacgacgacg atgtcgtcgg ggccacgccg

Example 10. Correlation between Gene Polymorphism of E-Selectin and glaucoma

Methods

Relationship between a E-selectin gene polymorphism and glaucoma among subject with POAG, NTG and normal subject was examined by means of Invader® method.

Invader® oligonucleotides (Invader® probe) used to detect the C/T polymorphism of SELE gene are shown in **Table 33-1.**

Table 33-1

Mutation	nucleotide change	Target	Probe	Sequence	Length (bp)	Tm (°C)	Dye
SELE 1402 CT	C to T	Anti-sense	Wild	Flap-CATGGATCAACTCAACTTGA	32	63.8	RED
			Mutant	Flap-TATGGATCAACTCAACTTGAG	31	63.4	FAM
			Invader	TCTTGTGCCTTCAGCTGTGAGGAGGGATTTGAATTAA	37	77.2	

Results

The 1402C>T polymorphism of E-selectin gene was confirmedbeing associated with both of POAG and NTG. Table 33-2) .

Table 33-2. E-selectin 1402C>T

	N	Genotype Frequency			p	Genotype Frequency		P	Genotype Frequency		χ^2 test p
		CC	CT	TT		CC	CT+TT		CC+CT	TT	
Control	224	138	67	19		138	86		205	19	
POAG	250	150	90	10		150	100		240	10	0.042
NTG	176	117	53	6		117	59		170	6	0.037

Partial nucleotide sequence of E-selectin comprising the targeted polymorohism is as follows:

SELE No. 1402(underlined) C>T

7561 tgtttttatt ttattttaag ataaaaagaa ctattgaaga gcttgggaac ttggttacct

7621 tgggaaacgt attgctggag atgcaaacaa acttctaaag tgctctctcg tgtgttccag

7681 ctgtgagatg cgatgctgtc caccagcccc cgaagggttt ggtgaggtgt gctcattccc

7741 ctattggaga attcacctac aagtcctctt gtgccttcag ctgtgaggag ggatttgaat

7801 tacatggatc aactcaactt gagtgcacat ctcagggaca atggacagaa gaggttcctt

7861 cctgccaagg tagaattgag tgcagacttt tttagggtac aggtcaaata cttcataaag

7921 tttctgaacc tagattgccc caaaggggtt tggtcctaat ttcctacatg ctgaaaacta

7981 agtagcgctt acactttaca ttcattgttg acttttaagc aagttttgga agttttccag

8041 tagatttttc tgaaactctg cctgtgtacc taacatttgc agtggtaaaa tgttcaagcc

8101 tggcagttcc gggaaagatc aacatgagct gcagtgggga gcccgtgttt ggcactgtgt

Example 11. Paraoxonase 1 gene polymorphisms are associated with clinical features of open-angle glaucoma

Purpose: Oxidative derivatives of low-density lipoprotein (LDL) are injurious to endothelium. Endothelial dysfunction

is known to be involved in the pathogenesis of open-angle glaucoma (OAG). High-density lipoprotein (HDL) prevents the oxidative modification of LDL. We examined whether polymorphisms in the paraoxonase 1 (PON1), PON2, and platelet-activating factor acetylhydrolase (PAF-AH) genes, HDL-associated antioxidant enzymes, were associated with OAG in a Japanese population.

MATERIALS and METHODS

Patients and control study subjects

Six hundred and ninety-eight blood samples were collected at seven Japanese institutions. Subjects included 190 POAG patients, 268 NTG patients, and 240 normal controls. None subject was related to any other.

Age at the blood sampling (mean ± SD) was 65.3 ± 11.9 years in POAG patients, 58.8 ± 13.4 years in NTG patients, and 69.7 ± 11.2 years in normal subjects, normal control subjects were significantly older than POAG patients ($p < 0.001$) or NTG patients ($p < 0.001$), which would reduce the likelihood of control subjects eventually developing glaucoma.

Clinical features recorded in glaucoma patients were age at diagnosis, IOP at diagnosis, and visual field defects at diagnosis. Severity of visual field defects was scored from 1 to 5. Data obtained with different perimeters were combined using a five-point scale defined as follows:

1 = no alternation; 2 = early defect; 3 = moderate defect; 4 = severe defect; 5 = light perception only or no vision. Field defects were judged to be early, moderate, or severe according to Kozaki's classification based on Goldmann perimetry or by the classification used for the Humphrey field analyzer. The former classification has been most widely used in Japan so far.

All patients received serial ophthalmic examinations including IOP measurements by Goldmann applanation tonometry, Humphrey perimetry (30-2) or Goldmann perimetry, gonioscopy, and optic disc examination including fundus photograph. All of glaucoma patients were diagnosed according to the following criteria: the presence of typical optic disc damage with glaucomatous cupping (cup/disc ratio >0.7) and loss of neuroretinal rim; reproducible visual field defects compatible with the glaucomatous cupping; and open angles on gonioscopy. Among the OAG patients, POAG was diagnosed if they had an IOP >21 mm Hg at any time during the follow-up period. Patients with exfoliative glaucoma, pigmentary glaucoma, and corticosteroid-induced glaucoma were excluded. Among the OAG patients, NTG was diagnosed when: the untreated peak IOP was consistently equal to or less than 21 mm Hg at all times including the 3 baseline measurements and that during the diurnal testing values (every 3 hours from 6 AM to 24

PM); the peak IOP with or without medication after diagnosis was consistently <22 mm Hg throughout the follow-up period; and the absence of a secondary cause for glaucomatous optic neuropathy, such as a previously elevated IOP following trauma, a period of steroid administration, or uveitis.

Control subjects were recruited from among Japanese individuals who had no known eye abnormalities except for cataracts. These subjects numbered 196 and were older than 40 years, with IOP below 20 mm Hg, no glaucomatous disc change, and no family history of glaucoma.

Genotyping

Genomic DNA was isolated from peripheral blood lymphocytes by standard methods. Four SNPs were then detected in all participants: two for *PON1* (L55M, Q192R); one for *PON2* (Cys311Ser, C311S); and one for *PAF-AH* (V279F).

These SNPs were genotyped by means of the Invader® assay (Third Wave Technologies, Inc, Madison, WI, USA) which was recently developed for high-throughput genotyping of SNPs. The oligonucleotide sequences of primary probes and Invader® probes used in this study were listed in **Table 34.**

Table 34. Sequences of primary probes and Invader oligonucleotides used in assays

Polymorphism	Nucleotide change	Target	Probe	Probe	Sequence
PON M55L	A to T	Sense	Wild	A probe	Flap sequences-TGTCTTCAGAGCCAGTT
			Mutant	T probe	Flap sequences-AGTCTTCAGAGCCAGTT
			Invader	Invader	AGAGCTAATGAAAGCCAGTCCATTAGGCAGTATCTCCAC
PON Q192R	A to G	Anti-sense	Wild	A probe	Flap sequences-AATCCTGGGAGATGTATTTG
			Mutant	G probe	Flap sequence-GATCCTGGGAGATGTATTTG
			Invader	Invader	AGCACTTTTATGGCACAAATGATCACTATTTTCTTGACCCTACTTACT
PAF-AH V279F	G to T	Sense	Wild	G probe	Flap sequences-CCGTTGCTCCACCA
			Mutant	T probe	Flap sequences-ACGTTGCTCCACCA
			Invader	Invader	ACTATCTTATTTTCTTACCTGAATCTCTGATCTTCACTAAGAGTCTGAATAAT

Statistical analysis

Hardy-Weinberg equilibrium was assessed by chi-squared analysis. Frequencies of the genotypes and alleles were compared between cases and controls by chi-squared analysis. Multivariate analyses were performed with a logistic regression model to confirm the association between the three clinical variables and the genotype.

Comparison of IOPs between genotype groups of Q192R in he PON 1 gene was performed by Kruskal-Wallis test. Statistical analyses were carried out with SPSS (version 12.0; SPSS, Chicago, IL). A value of $p < 0.05$ was considered to indicate significance.

RESULTS

Distributions of genotypes for the four SNPs in glaucoma patients and controls are shown in **Table 35**. The L55M polymorphism of the PON1 gene had a significantly different genotype frequency in patients with NTG.

Distribution of genotypes for polymorphisms in the PON2 gene and PAF-AH gene showed no significant differences between any patient group and controls (**Table 35**). And there was no significant difference in allele frequency of the 4 SNPs.

Table 35. Genotype frequency of PON1, PON2, and PAF-AH polymorphisms in Japanese control subjects and glaucoma patients

Phenotype	PON1/L55M				PON1/Q192R				PON2/C311S				PAF-AH/V279F			
	LL (%)	LM (%)	MM (%)	P	QQ (%)	QR (%)	RR (%)	P	CC (%)	CS (%)	SS (%)	P	VV (%)	VF (%)	FF (%)	P
Control	190	34	0		32	105	85		10	74	140		153	62	9	
(N=224)	84.8	15.2	0.0		14.4	47.3	38.3		4.5	33.0	62.5		68.3	27.7	4.0	
POAG	145	29	0		22	74	78		3	73	100		293	113	14	
(N=174)	83.3	16.7	0.0	0.922	12.6	42.5	44.8	0.421	1.7	41.5	56.8	0.093	69.8	26.9	3.3	0.874
NTG	224	19	3		44	100	102		9	88	151		121	48	5	
(N=246)	91.1	7.7	1.2	0.009	17.9	40.7	41.5	0.265	3.6	35.5	60.9	0.814	69.5	27.6	2.9	0.824

The distributions of the combined two polymorphisms of the PON1 gene in OAG population are shown in Table 36. As clearly shown, methionine (M) at position 55 (M allele) was rarely associated with arginine (R) at position 192 (R allele). Analysis confirmed a linkage disequilibrium between the polymorphisms giving rise to leuicine (L) at

position 55 and arginene (R) at position 192 (P<0.001).

Table 36. Distribution of genotypes defined by polymorphisms of PON1 gene affecting amino acids at position 55 and 192

		Q192R			
		QQ	QR	RR	Total
L55M	LL	72	221	265	558
	LM	23	58	0	81
	MM	3	0	0	3
	Total	98	279	265	642

		Q192R	
		Non R-carrier	R-carrier
L55M	L-carrire	95	544
	Non L-carrier	3	0

Characteristics of patients were examined in dominant and recessive models for each polymorphism. In the recessive model, no significant difference was seen in

three characteristics in patients with OAG for any polymorphisms. Significant differences with the dominant model of PON1 polymorphisms are shown in **Tables 37 and 38**. For L55M polymorphism in the PON1 gene in OAG patients, the LL group (non-55M carriers) was significantly younger at diagnosis than the LM+MM group (55M carriers) (56.8 ± 12.8 years vs. 60.1 ± 11.4, p=0.028) (**Table 37**). This association was not observed in POAG patients, but in NTG patients (55.6 ± 13.1 years vs. 63.7 ± 9.6, p=0.001).

For Q192R polymorphism, untreated maximum IOPs at diagnosis were significantly higher in OAG patients with QR+RR group (192R carriers) (21.5 ± 7.4 mm Hg) than those with QQ group (non-192Rcarriers) (18.7 ± 5.3 mm Hg, P=0.006, **Table 38**). Untreated maximum IOPs were higher in 192R carriers than in non-carriers among POAG patients (27.5 ± 7.0 mm Hg vs. 24.0 ± 4.9 for POAG, p=0.049) as well as among NTG patients (15.8 ± 2.8 mm Hg vs. 16.7 ± 2.4 for NTG, p=0.030).

Table 37 Clinical characteristics of NTG patients according to genotype of L55M in the PON1 gene

Phenotype	Clinical characteristics	Genotype		P value*
		LL	LM+MM	
OAG	Age at diagnosis (ys)	56.8 ± 12.8 (n = 473)	60.1 ± 11.4 (n = 62)	0.028
	IOP at diagnosis (mm Hg)	21.1 ± 7.2 (n = 409)	21.5 ± 6.1 (n = 58)	0.681
	Visual field score at diagnosis	2.9 ± 0.8 (n = 476)	3.0 ± 0.7 (n = 63)	0.899
POAG	Age at diagnosis (ys)	58.6 ± 12.2 (n = 199)	58.2 ± 12.3 (n = 34)	0.836
	IOP at diagnosis (mm Hg)	27.3 ± 7.1 (n = 170)	25.9 ± 4.8 (n = 31)	0.352
	Visual field score at diagnosis	3.9 ± 0.9 (n = 200)	3.0 ± 0.7 (n = 35)	0.475
NTG	Age at diagnosis (ys)	55.6 ± 13.1 (n = 274)	63.7 ± 9.6 (n = 28)	0.001
	IOP at diagnosis (mm Hg)	16.6 ± 2.5 (n = 239)	16.6 ± 2.7 (n = 27)	0.984
	Visual field score at diagnosis	2.8 ± 0.7 (n = 276)	2.9 ± 0.7 (n = 28)	0.343

P value* with Logistic regression analyses

Table 38 Clinical characteristics of glaucoma patients according to genotype of Q192R in the PON1 gene

Phenotype	Clinical characteristics	Genotype		P value*
		QQ	QR+RR	
OAG	Age at diagnosis (ys)	56.2 ± 13.9 (n = 77)	57.5 ± 12.4 (n = 468)	0.974
	IOP at diagnosis (mm Hg)	18.7 ± 5.3 (n = 66)	21.5 ± 7.4 (n = 409)	0.006
	Visual field score at diagnosis	2.7 ± 0.7 (n = 77)	2.9 ± 0.8 (n = 472)	0.100
POAG	Age at diagnosis (ys)	55.2 ± 12.8 (n = 29)	58.9 ± 12.0 (n = 210)	0.259
	Untreated IOP at diagnosis (mm Hg)	24.0 ± 4.9 (n = 23)	27.5 ± 7.0 (n = 183)	0.049
	Visual field score at diagnosis	2.8 ± 0.7 (n = 29)	3.1 ± 0.9 (n = 212)	0.415
NTG	Age at diagnosis (ys)	56.8 ± 14.6 (n = 48)	56.4 ± 12.7 (n = 258)	0.395
	Untreated IOP at diagnosis (mm Hg)	15.8 ± 2.8 (n = 43)	16.7 ± 2.4 (n = 226)	0.030
	Visual field score at diagnosis	2.7 ± 0.7 (n = 48)	2.8 ± 0.7 (n = 260)	0.155

P value* with Logistic regression analyses

The Gly192Arg (Q192R) polymorphism in PON1gene was associated with POAG (Table 39). The Leu55Met polymorphism was associated with NTG, especially with less than 15mmHg (Table 40)

Table 39 PON1 Gln192Arg (Q192R)

	N	Genotype Frequency			p	Genotype Frequency		p	Genotype Frequency		χ^2 test p
		QQ	QR	RR		QQ	QR+RR		QQ+QR	RR	
Cont-rol	224	32	107	85		32	192		139	85	
POAG	110	14	39	57	0.049	14	96	0.021	53	57	0.016
NTG	160	32	66	62		32	128		98	62	

Table 40 PON1 Leu55Met (L55M)

	N	Genotype Frequency			p	Genotype Frequency		p	Genotype Frequency		χ^2 test p
		LL	LM	MM		LL	LM+MM		LL+LM	MM	
Cont-rol	226	192	34	0		192	34		226	0	
POAG	110	97	13	0		97	13		110	0	
NTG	160	144	13	3	0.013	144	16		157	3	
H-NTG	122	111	10	1		111	11		121	1	
L-NTG	34	29	3	2	0.034	29	5		32	2	0.009

H-NTG: NTG patients with intraocular pressure at 16 mmHg-21mmHg.

L-NTG: MTG patients with maximal intraocular pressure at 15mmHg or less.

Conclusion: PON1 gene polymorphisms may influence features of Japanese patients with OAG, especially those with NTG.

Partial nucleotide sequence of Paraoxonase 1 gene containing the targeted polymorphisms is as follows:

PON1 Codon 55(underlined) TTG(Leu) to ATG(Met) (Leu55Met)

and

PON1 Codon 192(underlined) CAA(Gln) to CGA(Arg) (Gln192Arg)

1 agagcctcct agcccgtcgg tgtctgcgcc catcgatccc tttgtctatc cccgaccatg

61 gcgaagctga ttgcgctcac cctcttgggg atgggactgg cactcttcag gaaccaccag

121 tcttcttacc aaacacgact taatgctctc cgagaggtac aacccgtaga acttcctaac

181 tgtaatttag ttaaaggaat cgaaactggc tctgaagact tggagatact gcctaatgga

241 ctggctttca ttagctctgg attaaagtat cctggaataa agagcttcaa ccccaacagt

301 cctggaaaaa tacttctgat ggacctgaat gaagaagatc caacagtgtt ggaattgggg

361 atcactggaa gtaaatttga tgtatcttca tttaaccctc atgggattag cacattcaca

421 gatgaagata atgccatgta cctcctggtg gtgaaccatc cagatgccaa gtccacagtg

481 gagttgttta aatttcaaga agaagaaaaa tcgcttttgc atctaaaaac catcagacat

541 aaacttctgc ctaatttgaa tgatattgtt gctgtgggac ctgagcactt ttatggcaca

601 aatgatcact attttcttga cccctactta caatcctggg agatgtattt gggtttagcg

661 tggtcgtatg ttgtctacta tagtccaagt gaagttcgag tggtggcaga aggatttgat

721 tttgctaatg gaatcaacat ttcacccgat ggcaagtatg tctatatagc tgagttgctg

781 gctcataaga ttcatgtgta tgaaaagcat gctaattgga ctttaactcc attgaagtcc

841 cttgacttta ataccctcgt ggataacata tctgtggatc ctgagacagg agacctttgg

901 gttggatgcc atcccaatgg catgaaaatc ttcttctatg actcagagaa tcctcctgca

961 tcagaggtgc ttcgaatcca gaacattcta acagaagaac ctaaagtgac acaggtttat

Example 12. Evaluation of the *Noelin 2* gene in the ethiology of open-angle glaucoma

Purpose: To screen for mutations in the *Noelin 2* gene in Japanese patients with open-angle glaucoma using denaturing

high-performance liquid chromatography (DHPLC).

Methods

Subjects

A total of 616 blood samples were collected at eight institutions in Japan. There were 276 POAG patients, 340 NTG patients, and 300 normal controls, and none of the subjects was related to others in this study.

DNA Extraction and PCR Conditions

All of the blood samples were analyzed at Keio University. Genomic DNA was isolated from peripheral blood lymphocytes by phenol-chloroform extraction. The 6 exonic coding regions of the *Noelin 2* gene were amplified by polymerase chain reaction (PCR) using the primer sets listed in **Table 41.**

Table 41. Primer sequences, PCR product sizes, and PCR annealing and DHPLC analysis temperatures

Exon		Primer Sequences (5' to 3')	PCR product size (bp)	PCR Tm (°C)	DHPLC Tm (°C)
1	F	not determined			
	R	not determined			
2	F	GCGAGACCCTCACTGGGATT	344	67	62.0, 63.0, 64.0
	R	GCCTGGAGAGGAGCTGGATT			
3	F	GGTTGGGATTTGGGGAAGGA	284	67	60.3, 62.3, 64.3
	R	CCAGACATGACTCCATTGTAGGAA			
4A	F	GAGTCAGAGGTTGGAGTCATGT	249	65	62.7, 63.2, 63.7
	R	CCGTTGCTGCAGGTCCTCATA			
4B	F	CAGACACGCGGACCATTGTA	208	65	63.1, 64.1, 65.1
	R	GGGTGTGGCAGTCAGAGATCA			
5	F	CCCAACTTGATCACAGCACTT	269	65	61.7, 63.7, 64.7
	R	CTAGGCACCTATGGGCAGTCAA			
6A	F	CTAATGGCTGTAGCTGGTGCT	336	65	62.5, 63.5, 64.5
	R	GTAGGGGAAGGTGTTGTTGTAA			
6B	F	CCAGAGCAACGTGGTGGTCA	248	67	
	R	GGTAGCCGGTGTCCCAGGA			
6C	F	GGCTGTGTACACCACCAACCA	214	67	
	R	CTCGTAACTGGACGTGTTGGT			
6D	F	CATGATCTGCGGTGTGCTCTA	267	67	61.5, 62.0
	R	GCAGCCCGAGCCACAGCATT			

In high-throughput analysis, samples from three patients were pooled. PCR was performed with a thermal cycler (iCycler, Bio-Rad; Hercules, CA) in a total volume of 20 µl containing; 45 ng of genomic DNA, 2 µl GeneAmp 10x PCR buffer II, 2 µl of GeneAmp dNTP mix with a 2.0 mM concentration of each dNTP, 2.4 µl of a 25 mM $MgCl_2$ solution; 4 pmol of each primer, and 0.1 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA). The PCR conditions were; denaturation at 95° C for 9 min, followed by 35 cycles at 95° C for 1 min, 65° C or 67° C for 30 sec (Table 1), and 72° C for 1 min and 30 sec, and a final extension step at 72° C for 7 min.

Denaturing HPLC Analysis

For high-throughput analysis, a 25 µl volume of PCR products from the three patients was automatically injected into the chromatograph for analysis using the WAVE® System for DHPLC analysis (Transgenomic, Omaha, NE). The DHPLC melting temperatures are listed in **Table 41.**

When abnormal chromatographic patterns were detected in the pooled samples by the high-throughput protocol, the sample was reanalyzed individually in the WAVE® System. The PCR product that showed the abnormal chromatographic pattern was then sequenced.

Direct DNA Sequencing

For direct sequencing, PCR products were purified with a QIA Quick PCR purification kit (Qiagen, Valencia, CA) to remove unused primers and precursors. The PCR products were directly sequenced with the same forward and reverse PCR amplification primers on an ABI310 automated sequencer using BigDye chemistry according to the manufacturer's recommended protocol (Applied Biosystems, Foster City, CA).

Screening Myocilin Gene

Two patients with glaucoma who harbored the mutation in the Noelin 2 gene were screened in the myocilin gene by DHPLC.

Genotyping Noelin 2 c.462G>A (Arg144Gln) Polymorphism

The G to A substitution at position c.462 in exon 4 of the *Noelin 2* gene was detected by using restriction enzyme, BstU1. The G allele sequence was cut into two fragments (140 bp + 200 bp) by BstU1, while the A allele sequence remained intact (344 bp).

The polymorphism was confirmed by restriction-enzyme assay and by the chromatographic pattern of DHPLC.

Statistical Analyses

The frequencies of the genotypes and alleles in patients and controls were compared with the chi-square test or Fisher's exact test. The Hardy-Weinberg equilibrium for the observed frequencies was also calculated. Statistical analysis was performed with SPSS program (SPSS Inc., Chicago, USA). A *P* value of <0.05 was considered to be significant.

Results

Noelin 2 Variants in Japanese Subjects

A total of 616 Japanese subjects were studied, and the results are presented in **Table 42**. Ten sequence changes were identified in the glaucoma patients and control subjects. Among these, two were missense changes, seven were synonymous codon changes, and one was a change in intron sequences. One possible disease causing-mutation, Arg144Gln, was identified in one POAG proband and one POAG

proband, and was not present in the 300 normal Japanese controls. No significant difference was detected between glaucoma patients and controls for the Arg106Gln (P=0.30), Ala226Ala (P=0.30), and Arg427Arg (P=0.30).

The NTG patient with Arg144Gln harbored the Arg76Lys change in the myocilin gene.

A possible glaucoma-causing mutation in exon 4, Arg144Gln, was identified in 2(0.3%) of the 616 Japanese glaucoma patients.

Table 42. OLFM2 Variants oberved in glaucoma patients and control subjects

Location	Sequence Changes	Codon Changes	Frequency in Subjects (%) POAG	NTG	Control
Exon 4	c.462G>A	Arg144Gln	1 / 276 (0.4)	1 / 340 (0.3)	0 / 300 (0)
Exon 3	c.348G>A	Arg106Gln	111 / 211 (52.6)	135 / 276 (48.9)	115 / 241 (47.7)
Exon 3	c.289G>A	Thr86Thr	1 / 211 (0.5)	0 / 276 (0)	0 / 241 (0)
Exon 3	c.346G>A	Ala105Ala	1 / 211 (0.5)	0 / 276 (0)	0 / 241 (0)
Exon 4	c.451G>A	Lys140Lys	1 / 276 (0.4)	0 / 340 (0)	0 / 300 (0)
Exon 4	c.487G>A	Glu152Glu	2 / 276 (0.7)	0 / 340 (0)	0 / 300 (0)
Exon 5	c.628C>T	Thr199Thr	0 / 211 (0)	1 / 274 (0.4)	0 / 241 (0)
Exon 5	c.709G>A	Ala226Ala	15 / 211 (7.1)	27 / 274 (9.9)	28 / 241 (11.6)
Exon 6	c.1312C>T	Arg427Arg	34 / 211 (16.1)	45 / 270 (16.7)	30 / 240 (12.5)
Intron 6	c.1393+42T>C		117/210 (55.7)	N/C	N/C

* Sequence variation was found by direct sequencing analysis.

Partial nucleotide sequence of Noelin 2 comprising the targeted polymorphisms is as follows:

Noelin 2 codon 144 (underlined) CGG(Arg) to CAG(Gln) : (GG: 200 bp+144 bp, GA: 344 bp+200 bp+144 bp, AA: 344 bp) (BstUI)

codon 140(underlined) Lys140Lys (AAG>AAA)

codon 152(underlined) Glu152Glu (GAG>CAA)

79741 ttagttccta caatggagtc atgtctggga agaatctagg gtccaatatg agccacatgt

79801 caagggccag gtgtgcatca aagacaaagg gtgaagttat gagtcagagg ttggagtcat

79861 gtctgggtca aaggccaggg gtcaggcttg gccatggttc catcttgatg cacaggagct

79921 gaaggacagg atgacggaac tgttgcccct gagctcggtc ctggagcagt acaaggcaga

79981 cacgcggacc attgtacgct tgcgggagga ggtgaggaat ctctccggca gtctggcggc

80041 cattcaggag gagatgggtg cctacgggta tgaggacctg cagcaacggg tgatggccct

80101 ggaggcccgg ctccacgcct gcgcccagaa gctgggtatg ccttggccct tgaccctgac

80161 ccctgatctc tgactgccac acccaactcc agtatcacct gtttgtgcct agaagctgga

80221 cacagttttg acctctaact tttaaacctc aacccttgac cttcctacct aaggctacac

79841-79862, 80164-80184; primers for detecting polymorphism at codon 144

79916-80131, coding reagion

Example 13. Evaluation of the Heat shock protein 70-1(HSP70-1) gene in the etiology of glaucoma

Association between glaucoma and gene polymorphism of HSP70-1 (Biogerontology 4: 215-220, 2003 and Hum Genet 114: 236-241, 2004) was examined among POAG, NTG patients and control subject using Invader assay.

The primary probes (wild and mutant probes) and Invader® oligonucleotides (Invader® probe) used to detect the polymorphism of HSP70-1 gene are shown in **Table 43.**

Table 43. The oligonucleotide sequence of HSP70-1

Gene	Polymorphism	nucleotide change	format	Probe	Sequence
HSP70-1	-110A>C	A to C	PCR	A	Flap sequence-TTTTCGCCTCCCGT
				C	Flap sequence-GTTTCGCCTCCCGT
				Invader	GCTGCCAGGTCGGGAATATTCCAGGGC
			PCR	F	CGCCATGGAGACCAACACCC
				R	GCCGGTTCCCTGCTCTCTGTC

Results

As shown in Table 44, the polymorphism of -110A>C in HSP70-1 is associated with glaucoma, especially POAG.

Table 44. Genotype distribution and allele frequency of HSP70-1 gene polymorphisms in glaucoma patients and controls

HSP70-1 -110A>C	Genotype Frequency								Allele frequency			
	AA	AC	CC	p	AA	AC+CC	p	AA+AC	CC	p	A	C	p
CONTROL	67	130	44		67	174		197	44		264	218	
	27.8	53.9	18.3		27.8	72.2		81.7	18.3		64.8	45.2	
241	106	130	54	0.069	106	184	0.032	236	54	0.914	342	238	0.169
NTG	38.8	44.8	18.6		38.6	63.4		81.4	18.6		59.0	41.0	
290	84	94	33	0.026	84	127	0.007	178	33	0.460	262	160	0.026
POAG	39.8	44.5	15.8		39.8	60.2		84.4	15.6		62.1	37.9	
211	84	94	33	0.026	84	127	0.007	178	33	0.765	262	160	0.044
	39.8	44.5	15.8		39.8	60.2		84.4	15.6		62.1	37.9	
GLAUCOMA	190	224	87	0.020	190	311	0.007	414	87	0.765	604	398	0.044
501	37.9	44.7	17.4		37.9	62.1		82.6	17.4		60.3	39.7	

Partial nucleotide sequence of HSP70-1 comprising the targeted sequence is as follows:

HSP70-1 -110A>C (the following sequence is the C allele.)

1 cgccatggag accaacaccc ttcccaccgc cactcccccc tcctctcagg gtccctgtcc

61 cctccagtga atcccagaag actctggaga gttctgagca gggggcggca ctctggcctc

121 tgattggtcc aaggaaggct ggggggcagg acgggaggcg aaaccccggg aatattcccg

181 acctggcagc ctcatcgagc tcggtgattg gctcagaagg gaaaaggcgg gtctccgtga

241 cgacttataa aacgccaggg gcaagcggtc cggataacgg ctagcctgag gagctgctgc

301 gacagtccac taccttttc gagagtgact cccgttgtcc caaggcttcc cagagcgaac

Example 14. Evaluation of the Endothelin converting enzyme 1(ECE1) gene in the etiology of glaucoma

Association between glaucoma and gene polymorphism of ECE1 was examined in POAG and NTG patients using Invader assay.

The primary probes (wild and mutant probes) and Invader® oligonucleotides (Invader® probe) used to detect the polymorphism of ECE1 gene are shown in **Table 45.**

Table 45. The oligonucleotide sequence of ECE1

Gene	Polymorphism nucleotide change	Target	format	arm	Probe		Sequence	Length (bp)	Tm (°C)	Dye
ECE1	C-338A	Sense	PCR	1-3	C	Flap sequence-GTGGCCCAGAGCA		23	63.0	FAM
					A	Flap-sequence-TTGGCCCAGAGCAA		26	63.2	RED
					Invader	GGCAGATAACAAAAGTATCAGGAAGGTGCCCTCGATC		37	77.5	
			PCR		F	TAAGTCCCCCTTCAACAACC				
					R	AAGCTGAAAAGTACGCATAAATG				

Results

As shown in Table 46, the polymorphism of -338C>A in ECE1 is associated with high IOP in NTG.

Table 46. Genotype distribution of ECE-1 gene polymorphisms in glaucoma patients and controls

ECE-1/-338C>A polymorphism		three genotypes									two genotypes								
	Clinical chracteristics	CC		n	CA		n	AA		n	p	CC+CA		n	AA		n	p	
POAG	Age at diagnosis (ys)	56.8	± 12.2	68	57.8	± 12.4	106	61.9	± 10.5	34	0.089	57.4	± 12.3	174	61.9	± 10.5	34	0.032	
	IOP at diagnosis (mm Hg)	26.2	± 5.8	60	26.8	± 6.5	94	26.6	± 4.8	32	0.301	26.5	± 6.2	154	26.6	± 4.8	32	0.285	
	Visual field score at diagnosis	3.1	± 1.0	68	3.1	± 0.9	105	3.0	± 0.8	35	0.917	3.1	± 0.9	173	3.0	± 0.8	35	0.761	
NTG	Age at diagnosis (ys)	59.1	± 13.0	97	54.2	± 12.2	136	54.1	± 14.2	53	0.015	56.2	± 12.7	233	54.1	± 14.2	53	0.350	
	IOP at diagnosis (mm Hg)	16.7	± 2.4	91	16.8	± 2.4	123	15.6	± 2.6	46	0.024	16.7	± 2.4	214	15.6	± 2.6	46	0.007	
	Visual field score at diagnosis	2.8	± 0.7	99	2.8	± 0.7	136	2.8	± 0.7	53	0.704	2.8	± 0.7	235	2.8	± 0.7	53	0.534	

ECE-1/-338C>A polymorphism		two genotypes							
	Clinical chracteristics	CC		n	CA+AA		n	p	
POAG	Age at diagnosis (ys)	56.8	± 12.2	68	58.8	± 12.1	140	0.262	
	IOP at diagnosis (mm Hg)	26.2	± 5.8	60	26.7	± 6.1	128	0.161	
	Visual field score at diagnosis	3.1	± 1.0	68	3.0	± 0.9	140	0.715	
NTG	Age at diagnosis (ys)	59.1	± 13.0	97	54.1	± 12.8	189	0.004	
	IOP at diagnosis (mm Hg)	16.7	± 2.4	91	16.5	± 2.5	169	0.507	
	Visual field score at diagnosis	2.8	± 0.7	99	2.8	± 0.7	189	0.755	

Partial nucleotide sequence of ECE-1 comprising the targeted polymorphism is shown as follows:

ECE1 -338C>A (underlined)

1 ttttgtctgg tctttctagc attaaccccc tagacacacc taaggctgat gccgggggga

61 acctgtcttg attgctctgg gccacatcga gggcaccttc ctgatacttt tgttatctgc

121 cactggggac ccggttgttg aaggggggact taagattttc tcgaaggagg ggtcactgtg

181 agggcctttc ctgcctgcta ggggcttcag tttgggggcc cccactcccg actccgggca

241 agggaggggt ccccatctcc cccgggcctc tcgggtcttg gggtctcccc gggaggccgg

Example 15. Evaluation of the CD50 gene in the etiology of open-angle glaucoma

Polymorphism of CD50 gene was identified using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) techniques (**Table 47**).

Table 47. Primer sequences, product size, and annealing temperatures

Gene		Primer sequences (5' to 3')	primer name	Product size (bp)	Annealing temperature (°C)	Restriction Enzyme
CD95 (A-670G)	F	CTA CCT AAG AGC TAT CTA CCG TTC	CD95F	232	65.0	Mva I
	R	GGC TGT CCA TGT TGT GGC TGC	CD95R			

Results

As shown in Table 48, the polymorphism of A-670G in CD95 is associated with glaucoma, especially POAG.

Table 48. Genotype distribution and allele frequency of CD95 gene polymorphisms in glaucoma patients and controls

CD95 A-670G	Genotype Frequency									Allele frequency		
	A/A	A/G	G/G	p	A/A	A/G+G/G	p	A/A+A/G	G/G	p	A	G
CONTROL	60	113	68		60	181		173	68		233	249
	24.9	46.9	28.2		24.8	75.1		71.8	28.2		48.3	51.7
NTG	69	145	76	0.769	69	221	0.768	214	76	0.604	283	297
	23.8	50.0	26.2		23.8	76.2		73.8	26.2		48.8	51.2
POAG	45	125	41	0.024	45	166	0.370	170	41	0.029	215	207
	21.3	59.2	19.4		21.3	78.7		80.6	19.4		50.9	49.1

Example 16. Evaluation of the EPHX1 gene in the etiology of glaucoma

Association between glaucoma and gene polymorphism of EPHX1 was examined among POAG, NTG patients and control

subject using Invader assay.

The primary probes (wild and mutant probes) and Invader® oligonucleotides (Invader® probe) used to detect the polymorphism of ECE1 gene are shown in **Table 49.**

Table 49. The oligonucleotide sequence of

Mutation	nucleotide change	Target	Probe	Sequence	Length	Tm	Dye
EPHX1 K119	G to A	Sense	Wild	Flap sequence-CTTAGTCTTGAAGTGAGGG	29	62.7	FAM
			Mutant	Flap sequence-TTTAGTCTTGAAGTGAGGG	31	62.3	RED
			Invader	TCTCTGGCTGGCGTTTTGCAAACATACCTTCAATA	35		

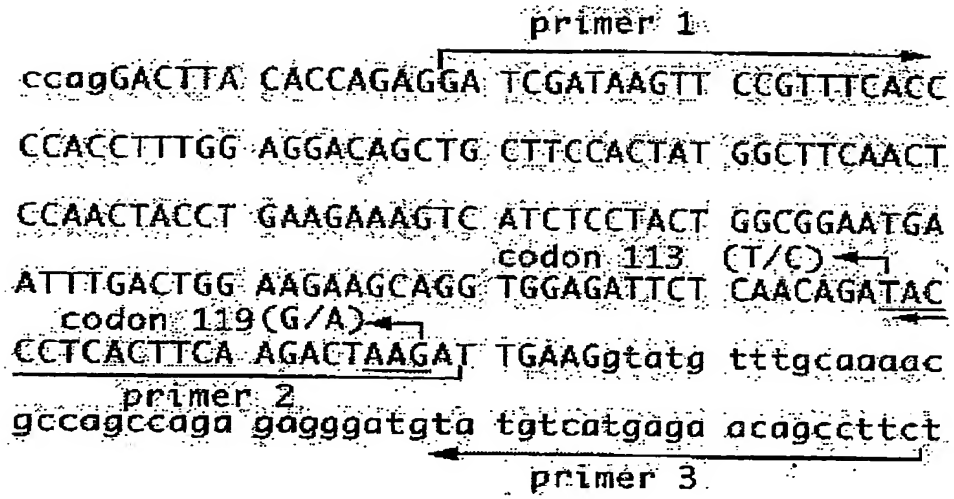
Results

As shown in Table 50, the polymorphism of G>A in codon 119 Lys is associated with glaucoma, especially NTG.

Table 50. Genotype distribution and allele frequency of EPHX1 gene polymorphisms in glaucoma patients and controls

EPHX1 G>A (Lys119Lys)	Genotype Frequency									Allele frequency		
	G/G	G/A	A/A	p	G/G	G/A+A/A	p	G/G+G/A	A/A	p	G	A	p
CONTROL	107	87	30		107	117		194	30		301	147	
	47.8	38.8	13.4		47.8	52.2		86.6	13.4		67.2	32.8	
224				0.100			0.891			0.039			0.286
NTG	121	110	19		121	129		231	19		352	148	
	48.4	44.0	7.6		48.4	51.6		92.4	7.6		70.4	29.6	
250				0.669			0.904			0.388			0.583
POAG	83	64	29		83	93		147	29		230	122	
	47.2	36.4	16.5		47.2	52.8		83.5	16.5		65.3	34.7	
176													

Partial nucleotide sequence of EPHX1 comprising the targeted polymoprhisms is as follows:



Example 17. Evaluation of the β2 adrenergic receptor (ADRB2) gene in the etiology of glaucoma

Association between glaucoma and gene polymorphism of ADRB2 was examined in open angle glaucoma patients (POAG and NTG patients) using Invader assay.

The primary probes (wild and mutant probes) and Invader® oligonucleotides (Invader® probe) used to detect the polymorphism of ADRB2 gene are shown in Table 51.

Table 51. The oligonucleotide sequence of ADRB2

Gene	Mutation	nucleotide change	Target	Probe	Sequence	Length (bp)	Tm (°C)	Dye
ADRB2	Gln16Arg (G46A)	G to A	Sense	A	Flap sequence-TATTGGGTGCCAGCA	27	63.8	RED
				G	Flap sequence-CATTGGGTGCCAGC	24	63.2	FAM
				Invader	TCGTGGTCCGGCGCATGGCTTCA	23	77.5	
ADRB2	Gln27Glu(Q79G)	C to G	Anti-Sense	C	Flap sequence-CAAAGGGACGAGGTGT	26	63.8	RED
				G	Flap sequence-GAAAGGGACGAGGTGT	30	63.4	FAM
				Invader	GCCGGACCACGACGTCACGCAGT	23	77.0	

Results

As shown in Table 52, the polymorphism of Gly16Arg(G46A) of ADRB2 is associated with early onset of POAG.

Table 52. Clinical characteristics of glaucoma patients according to genotype of Gln16Arg in the ADRB2 gene

ADRB2	Gly16Alg			
		Genotype		
Phenotype	Clinical characteristics	RR	RG+GG	*P* value*
OAG	Age at diagnosis (ys)	57.9 ± 12.7 (n =100)	56.3 ± 12.7 (n =371)	0.085
	IOP at diagnosis (mm Hg)	20.3 ± 5.8 (n = 90)	20.8 ± 6.5 (n = 335)	0.469
	Visual field score at diagnosis	2.8 ± 0.7 (n = 99)	2.9 ± 0.8 (n = 375)	0.508
POAG	Age at diagnosis (ys)	62.9 ± 12.7 (n = 39)	56.7 ± 11.7 (n = 162)	<0.001
	IOP at diagnosis (mm Hg)	26.3 ± 4.9 (n = 33)	26.3 ± 6.0 (n = 147)	0.973
	Visual field score at diagnosis	3.0 ± 0.9 (n = 38)	3.1 ± 0.9 (n = 164)	0.898
NTG	Age at diagnosis (ys)	54.7 ± 11.7 (n = 61)	56.0 ± 13.5 (n = 209)	0.531
	IOP at diagnosis (mm Hg)	16.8 ± 2.5 (n = 57)	16.6 ± 2.4 (n = 188)	0.581
	Visual field score at diagnosis	2.7 ± 0.5 (n = 61)	2.8 ± 0.7 (n = 211)	0.266

P value* with Logistic regression analyses

As shown in Table 53, the polymorphism of Gln27Glu(C79G) is associated with high intraocular pressure(IOP) in OAG, especially POAG.

Table 53. Clinical characteristics of glaucoma patients according to genotype of Gln27Glu in the ADRB2 gene

ADRB2	Gln27Glu(Q27E)			
	Phenotype Variable	QQ	QE+EE	P value*
POAG	Age at diagnosis (ys)	58.4 ± 12.3 (n = 162)	56.3 ± 12.2 (n = 30)	0.272
	IOP at diagnosis (mm Hg)	26.0 ± 5.1 (n = 144)	28.6 ± 9.1 (n = 28)	0.038
	Visual field score at diagnosis	3.1 ± 0.9 (n = 163)	3.1 ± 0.9 (n = 30)	0.837
NTG	Age at diagnosis (ys)	55.6 ± 12.8 (n = 250)	58.2 ± 12.6 (n = 23)	0.986
	IOP at diagnosis (mm Hg)	16.6 ± 2.5 (n = 230)	17.1 ± 2.0 (n = 17)	0.447
	Visual field score at diagnosis	2.8 ± 0.7 (n = 251)	2.8 ± 0.6 (n = 24)	0.692
OAG	Age at diagnosis (ys)	56.7 ± 12.7 (n =412)	57.1 ± 12.3 (n = 53)	0.448
	IOP at diagnosis (mm Hg)	20.2 ± 5.9 (n = 374)	24.2 ± 9.2 (n = 45)	<0.001
	Visual field score at diagnosis	2.9 ± 0.8 (n = 414)	2.9 ± 0.8 (n = 54)	1.000

*P value with Logistic regression analyses

Partial nucleotide sequence for ADRB2 gene containing the targeted polymorphisms is as follows:

ADRB2 codon Nos. Gly16Arg(GGA>AGA): Gln27Glu (CAA>GAA) (underlined)

1 gcgcttacct gccagactgc gcgccatggg gcaacccggg aacggcagcg ccttcttgct

61 ggcacccaat ggaagccatg cgccggacca cgacgtcacg cagcaaaggg acgaggtgtg

121 ggtggtgggc atgggcatcg tcatgtctct catcgtcctg gccatcgtgt ttggcaatgt

181 gctggtcatc acagccattg ccaagttcga gcgtctgcag acggtcacca actacttcat

241 cacttcactg gcctgtgctg atctggtcat gggcctagca gtggtgccct ttggggccgc

301 ccatattctt atgaaaatgt ggacttttgg caacttctgg tgcgagtttt ggacttccat